

Criteria for science in the courts

The US Supreme Court may produce new criteria for the admissibility of scientific evidence in the courts on the basis of a suit now being heard.

THE question of what constitutes valid scientific data, suitable for admission as evidence in court, has plagued judges for decades. Generally unschooled in the scientific method, judges have the legal duty of deciding what may or may not be presented to a jury. For years, US judges have relied on a standard, dating back to 1923 and too often honoured in the breach, defining admissible evidence as that which derives from methods of inquiry that are 'generally accepted' by the scientific community. Frequently, courts have interpreted that to mean 'published in the peer-reviewed literature'.

A more lenient standard, set out in legislation in 1975, permits judges (at their discretion) to admit as evidence almost any opinion from an 'expert witness', defined as someone who is qualified "by knowledge, skill, experience, training or education" to speak to a given subject. Each standard is in some way deficient.

First, it is (or should be) well known that the peer review system is not infallible and, further, that the best journals openly acknowledge that editorial judgement on the importance of a paper and its estimated interest to readers play an important role in deciding which papers to publish and which to reject. And even this journal has rejected papers that subsequently proved to be of exceptional significance. Thus to bar from the courts data that have not appeared in a peer reviewed journal could be foolhardy. But it is also well known that the so-called expert witness in court may be a hired gun, willing to testify to anything for a fee, or a crackpot whose unsupportable ideas are masked by an advanced degree — often from a respectable university.

The issue of standards of evidence arises now because of a case just argued before the US Supreme Court over whether data do or do not support the allegation that a drug called Bendectin, once widely prescribed to prevent morning sickness in pregnant women, causes limb deformities in newborn babies. The manufacturer and the defendant in the case, Merrell Dow (now Marion Merrell Dow of Kansas City), has consistently won its case in some 200 lawsuits brought by parents who claim that Bendectin is a teratogen. The company can cite more than 25 published epidemiological studies indicating no correlation between Bendectin (which was taken by more than 30 million women worldwide) and limb deformities. (Nevertheless, because of the high cost of litigation, the company withdrew Bendectin from the market in 1983, leaving women to rely on old-fashioned remedies to prevent what is, in some instances, a serious complication of pregnancy.)

The plaintiffs in earlier cases and that now before the Supreme Court, known as *Daubert v. Merrell Dow Pharmaceuticals*, have relied largely on the testimony of expert witnesses, some of whom have reached conclusions by analogy rather than direct experiment. Most of the time, the courts have ruled their testimony inadmissible. The issue has been cast in scientific circles and the press as a clash between 'good' science and what is scornfully described as 'junk' science because it fails to meet tests of scientific legitimacy. For instance, much of the case against Bendectin in *Daubert* rests on testimony by a Berkeley-trained epidemiologist, now affiliated with the California state health department, who claims that her "reanalysis" of the published epidemiological data shows a one in 1,000 incidence of limb deformities caused by Bendectin. She has not written up her data for publication.

What should the court do? Reflecting a befuddlement judges often express when dealing with science (and revealing again that science is not yet part of the mainstream of education) one of the justices said: "There are Harvard law professors on both sides of this case; I had hoped you could get together and lead us out of the wilderness." But it is not really a wilderness, as many of the 'friend of the court' briefs filed by scientific bodies suggest. One in particular (from the not-for-profit Carnegie Commission on Science, Technology and Government) offers a clear way out. The commission urges the justices to adopt a new standard for evidence that would require judges not to resolve scientific controversy but only to ask three pertinent questions in weighing admissibility of evidence: is the claim testable? Has it been tested? And is the methodology sound?

Courts should not exclude evidence just because it is not accepted wisdom; nor should they allow plaintiffs to be held liable on the basis of mere hypothesis or speculation. While it is true that speculation is an essential part of science, and true that new ideas may have a hard time gaining acceptance, it does not follow that untested science belongs in court. That would be bad public policy.

An influential fellow

The death two weeks ago of Lord Zuckerman will leave a sad gap in public life in Britain and elsewhere.

SOLLY (as even his enemies called him) Zuckerman, more formally Lord Zuckerman, OM, was an iconoclast by



temperament, a Pooh Bah by habit and a mandarin by instinct. Over nearly half a century, he was a powerful influence on several aspects of British public life, but mostly behind the scenes. Only in the past two decades (he was 88 when he died last week) has he spoken out in public, and in the liberal persuasion, on matters such as disarmament and Britain's policy on nuclear weapons, matters that occupied a great deal of his professional life in government. One of his last articles appeared earlier this year in *Nature* (361, 392; 4 February 1993) but the British government has not yet deigned to answer the awkward questions he raised in it.

Solly, Jewish and proud of it, and South African by origin, was an iconoclast in the limited sense that he saw it as his duty within the government and outside to tell people, officials and politicians when he considered that they were wrong. (But in the 1930s, while a teacher of anatomy at the University of Birmingham and investigating hormonal influences in reproduction, he had already been reproved for obscenity by the Archbishop of Canterbury — the executive head of the Anglican Church — over his first book, *The Social Life of Monkeys and Apes*.) His continued scepticism about Edward Teller's various proposals for innovation in nuclear weaponry and strategy became a legend; Teller was a friend he never made. But Zuckerman had a knack, a blend of humour and guile, that allowed him to tell his political masters they were mistaken without giving permanent offence. It was in that spirit that he persuaded Lord Mountbatten in the early 1960s to take Pugwash seriously. On another occasion, he dissuaded Mountbatten from listening to a hare-brained proposal for a military take-over of the government.

Zuckerman was not an institutional iconoclast, but sometimes the opposite. His appointment in 1955 as Secretary of the Zoological Society of London, for example, was marked by his affection for the zoo, but also by ructions as he fought the old guard for his sensible proposals for change. (Recent events have sadly shown that they were not radical enough; Zuckerman's regret a few weeks ago was that he was not still in charge.) His capacity for doing several jobs, and simultaneously, seems to have been formed during this period; he was essentially full-time executive of the London Zoo and its appurtenances while moving from the Ministry of Defence to the Cabinet Office as the most senior and confidential adviser in sight, whence the reference to Pooh Bah.

Zuckerman was a mandarin (the British name for the head of a government department) in the antique Chinese sense; he conveyed the sense of having no power personally, but that there was a powerful emperor over his shoulder. That, of course, is a recipe for survival, but there is no doubt that Zuckerman enjoyed the influence this gave him, outside government as within. He was a great manipulator. Even so, it is a pity that he did not accept the challenge offered by Harold Wilson, 1964's new Prime Minister, of a post in the Foreign Office with responsibility for disarmament. His most fitting memorial would be that his friends should now press for an answer to the awkward questions on nuclear policy the British government has not yet answered. □

Horse-racing hoax

The British, once a proud nation, have now lost even the trick of organizing horse races, preferring hoaxes instead.

ONCE upon a time, the British used to boast of the battles they had won — Agincourt, Trafalgar and Waterloo (with less justice), for example. Then they boasted about technological innovations — steam engines, railway locomotives, steam turbines and so on. Latterly, they have been reduced to boasting of the spectacles they organize — the Highland Games (in Scotland in the summer), the Oxbridge boat race on the Thames and the horse race called the Grand National, which should have taken place last Saturday.

As is widely known, the race never happened. Instead, the organizers staged a hoax on the 39 horses and their jockeys, together with their trainers, owners and other hangers-on, the few tens of thousands of people waiting in the rain at Aintree (near Liverpool), those who had wagered £75 million on the outcome of the race in Britain alone and the score of broadcasting organizations elsewhere that had bought the right to show the event on television. Is it possible that the organizers had misread the calendar, believing that Saturday was the first of April, not the third?

The centrepiece of the hoax was a mechanical device — a traditional way of arranging when horses in a race should begin to run. At most racetracks, horses are put in separate boxes which all open at the front when the race begins. Not so at the Grand National, where the horses are assembled behind a fibre rope (called a tape) some 70 metres long, whose ends are hoisted when the race is signalled to begin.

The technology of the starting mechanism is deceptively simple. Like any rope suspended from its ends, the Aintree tape must be disposed in catenary form. To allow the horses and their appended jockeys through, all points of the tape must rise by about 1 metre in the time it takes a horse to travel about the same distance from rest, perhaps much less than 0.1 seconds. Whether this will happen will be a function of the wave velocity along the tape, whose mass per unit length will have been increased, and wave velocity reduced, by the drenching rain last Saturday. On the first false start, the tape caught round some horses' necks. On the second, some jockeys were similarly incommoded.

The traditional technology for recalling horses after a false start is equally sophisticated: the official at the starting line waves a red flag if a start is false, and then a second official 200 metres down the track waves another. (Jockeys do not often look backwards at the beginning of a race.) Those who believe that klaxon horns or traffic lights (as used elsewhere), would more securely give warnings overlook the long tradition of the British flagman, one of whom by law carried a red flag before the early railway locomotives and then the first motor-cars. Aintree says puckishly that this arrangement is reliable because it depends on people's judgement, but it is also a hoaxer's charter. On Saturday, the device did not work the second time. It remains to be told who played the hoax, and why. No other explanation is charitable. □

AIDS administrators plan crisis meeting over future of AZT

London & Washington. Senior research administrators from both sides of the Atlantic plan to meet before the end of the month to discuss the implications of Anglo-French trial results which last week cast doubt on the usefulness of the drug AZT in treating HIV.

According to preliminary results published in *The Lancet*, the three-year trial, organized jointly by Britain's Medical Research Council and the French National AIDS Research Agency, has shown no evidence that treatment with AZT delays the onset of AIDS in patients known to be carrying the HIV virus.

The study was based on 1,749 patients. Some of these were given AZT from the start; the others were given a placebo until they developed symptoms of either AIDS or AIDS-related complex (ARC), when they were then switched to AZT. There was no significant difference in the rate at which AIDS progressed in the two groups; about 8 per cent in each group had died within the three years.

Scientists on both sides of the Atlantic have said that the so-called "Concorde" trial confirms what many had felt for a long time, namely that AZT taken on its own is likely to be limited in delaying the onset of AIDS. They hope that a combination of drugs — including AZT — will prove more effective, impelling mutations of the virus in inconsistent directions.

But differences of opinion have emerged on the regulatory significance of the Concorde trial. Some European scientists have criticized the decision of the US Food and Drug Administration, under heavy pressure from AIDS activists, to endorse the widespread use of AZT in pre-symptomatic AIDS patients on the basis of early results from four US studies showing the drug's apparent effectiveness.

The European critics feel that the results of the latest trials justify their initial scepticism about some of the early claims made as a result of the US studies.

Tony Pinching, professor of immunology at St Bartholomew's Hospital Medical College in London, acknowledges that the US studies showed a "possible benefit" to those infected with HIV. But he argues that the benefits were "too small and too short-term for us to know what they meant in the long-term". The results of the Concorde trial came as "a considerable shock", he says, particularly to those who had been expecting some positive outcome, however small.

In the United States, senior health administrators stressed that their advice to doctors and researchers to continue working with AZT in the immediate wake of the

Concorde results did not imply any criticism of the European study. "I have every reason to believe that this is a first rate study", says Daniel Hoth, AIDS director of the National Institute for Allergy and Infectious Diseases (NIAID). "The results need not be inconsistent with other trials. In the end we all agree that AZT is an effective drug, but only for a short period."

Hoth, who called the upcoming meeting with the Concorde group as soon as the results were published, reacted angrily to UK press criticism that previous US AZT trials had been rushed, pointing out that the trials were curtailed only because regulators judged, once AZT appeared to be inhibiting AIDS, that their continuation was unfair to patients on the placebo side of the trial.

Wellcome Foundation, which currently sells more than £200 million (\$300 million) of AZT a year, dismissed the use of AZT on its own as "old science", but also claimed that the Concorde conclusions were not as black-and-white as they appeared. But Gregg Gonsalves of the Treatment Action Group,

an AIDS activist organization in New York, said that large numbers of patients newly identified as HIV carriers were still being prescribed AZT on its own.

One matter to be discussed when the research administrators meet will be the Concorde trial's demonstration that the level of CD4 white blood cells rose significantly in patients who had been receiving AZT, even though there was no subsequent clinical benefit.

US regulatory officials have been using the level of CD4 (T-helper) cells as a surrogate measure of the progress (and alleviation) of AIDS symptoms. However, there are many in Britain who feel that the US approach, which has relied heavily on the CD4 measure in licensing other potential anti-AIDS drugs, is wrong, and that the decision to put a patient on AZT should be more broadly based, using a number of factors — including the best current estimate of likely long-term risks and benefits — rather than a simple formula.

David Dickson & Colin Macilwain

Rules eased for US field tests

San Francisco. The US Department of Agriculture (USDA), ending years of debate, has relaxed regulatory supervision of field tests of six important genetically engineered crops.

A new rule published on 31 March in the *Federal Register* exempts the crops from the long process required for a permit and allows biotechnology companies to carry out experiments with corn, cotton, tomatoes, soybeans, tobacco and potatoes after notifying USDA in a letter and waiting 30 days. The new rule would have allowed 85 per cent of the 365 permits issued for field tests at 724 sites to have gone forward under the simpler notification.

Biotechnology executives see the plan as a substantial improvement on the present system, in which the government analyses every proposed planting in a process that can take four months. The new rule is also a clear victory for environmentalists against a proposal by the Bush administration that would have eliminated any waiting period. Terry Medley, chief of the agriculture department's biotechnology section, points out that the rule is consistent with President Bill Clinton's intent to balance economic stimulation with environmental safety.

The preamble to the rule advises the industry to expect an additional review of genetically engineered pesticides and the commercialization and marketing of foods.

The Food and Drug Administration has also hinted that it may revise a policy that does not at present require special safety testing or labelling for most biotechnology foods.

The new field-testing rule requires regulators to tell researchers within a month whether they can proceed. Plants used to produce drugs are not eligible for the one-month notification. The new rule also allows the federal government to consult state officials on any regional scientific concerns.

A company may request a one-time exemption for a crop not on the list or it may ask the government to add its crop to a list of those permanently exempted from the permitting requirement. The rule abandons an earlier plan that would have allowed safety boards or state officials to help researchers make such decisions on their own.

Medley said that his agency received 84 comments from universities, states, public-interest groups and others on the proposed rule. The Industrial Biotechnology Association says that the 30-day plan reduces the chances that an increasing number of applications will clog the system. At the same time, it said, the new rule may help to build public trust by retaining clear federal supervision and by providing enough time for local officials and public interest groups to react to any proposed field test.

Sally Lehrman

British aerospace industry wants more government support

London. Britain's aerospace manufacturers, hoping to persuade government officials of the need to support key technologies to counter growing US competition, have asked the Department of Trade and Industry (DTI) to back a national research programme in advanced civil aeronautics research. In particular, they want the DTI to increase its support for such research by a factor of four, from £26 million (US\$38 million) to £100 million a year.

The proposal, drawn up by an advisory group of industrialists and academics known as the DTI's aviation committee, was originally put to the department last November but has only just been made public. It argues that a "national strategic technology acquisition plan" is needed to provide a boost to the industry's own research efforts comparable to what US companies receive from the National Aeronautics and Space Administration (NASA), which spends about \$900 million a year in the field. Britain has historically provided substantial direct aid for civil aviation research, most recently for the development of Airbus, but that support has ended as the programme approaches commercial viability.

"We want a plan formulated jointly by government, industry, research establishments and the academic community, which will prevent us all going off in different

directions", says John Stollery, professor of aeronautics at the Cranfield Institute of Technology and chairman of the committee. According to Stollery, there are many areas of aviation technology, ranging from aero-engines to advanced avionics systems, in which Britain is a world leader. "If we are to maintain this type of lead, we need realistic support similar to our competitors", he says.

So far, the DTI has shown little enthusiasm for the idea, and the budget for civilian aerospace research is scheduled to decline next year by 15 per cent, to £22 million. Aerospace officials, however, are hoping to change the government's mind by pointing to the new activist policies of President Bill Clinton and by citing evidence that the US government is spending considerably more than the three per cent of turnover accepted as a limit under a trade agreement signed with the European Commission last July.

Last year's expenditures by NASA of \$902 million barely exceeds three per cent of the US civil aviation industry's sales last year of \$29 billion. But Clinton has proposed that NASA spend an additional \$550 million over the next four years on civil aviation research, raising the government's level of support beyond the limit if, as seems likely, sales remain stagnant.

At present, the aeronautics and pharmaceutical industries make the largest positive contributions to Britain's balance of payments, capturing almost 12 per cent of the world's market for its products. But the committee warns that the industry's long-term future is threatened by a squeeze in defence spending and reduced civilian orders, and that its survival depends upon increased spending on long-term research.

Additional government spending is particularly important in such areas as advanced wing design, air traffic management systems and low-cost manufacturing. Aerospace officials say that support is growing for a national strategic plan in which increased government spending would be matched by the industry's own resources.

"Our message to the Treasury is that they have to look at public spending on research and development in aeronautics as an investment, not just an expenditure", says an official of the Society of British Aerospace Companies. "Our members feel that failure to sustain adequate R&D investment to counter international competition could mean an inevitable erosion of the UK's capability — and once lost, the industry's leading position could not be regained."

David Dickson

ESA is wary about impact of redesigned US station

Munich. The European Space Agency (ESA), whose pressurized space module Columbus is an ECU2.4-billion (US\$3.1 billion) part of the \$30-billion US space station Freedom, is considering other alliances in the wake of yet another redesign of the US station and amid fears that the US Congress may reject even a smaller, cheaper version.

ESA's contribution to the US station has been withheld until concrete proposals emerge from the redesign ordered last month by US president Bill Clinton, who instructed the National Aeronautics and Space Administration (NASA) to scale down the station and cut its operational costs in half (see *Nature* 361, 195; 1993). The fortunes of manned space travel have had a rough ride in the past few years, with the United States finally joining Europe in questioning the costs of such programmes in times of recession. ESA abandoned its own plans for independent manned missions last year, after much internal strife, but its commitment to Columbus was reaffirmed last November despite a funding

shortfall of 10 per cent.

ESA has already spent millions of dollars on Columbus, and it now seems likely that the redesign of Freedom will require a corresponding change in its space laboratory. But at least ESA will now have a voice. Europe and Japan were dismayed and angered last month by comments from the US government that ignored their significant roles in the programme (Japan is contributing a similar module). But since then NASA has promised to protect their interests (around 20 per cent of the programme's total cost), and both Europe and Japan are now represented in the discussions.

The deadline is very short. Clinton wants to see plans for the modified programme in early June, and a first draft is expected by mid-May. An outside advisory group, led by Charles Vest, president of the Massachusetts Institute of Technology, will provide a second layer of review.

In the meantime, ESA and its member countries are considering back-up plans for Columbus should the United States

abandon Freedom completely. ESA acknowledges its disappointment that the programme is being redesigned "at a point when we were almost ready to cut iron with our contribution", according to a spokesman. "Redesigning our part will cost us more money." But Germany, which led the campaign to reduce spending on space to help pay for the soaring cost of reunification, sees the situation as an opportunity to reduce the cost of Columbus as well.

Germany's new minister for science and technology, Matthias Wissmann, thinks that another way to save money is to merge Russia's MIR 2 programme with NASA's Freedom. But experts say such a proposal would not be technically feasible.

It might, however, be possible to combine the Japanese and European modules. But such an alliance is unlikely given each country's desire to retain control over its own module. Despite the behind-the-scenes politics, the most likely outcome of a redesigned Freedom is a slowing of ESA's programme to spread the costs over a longer period.

Allison Abbott

European labs begin parallel processing computer project

Munich. The European particle physics laboratory CERN and the European centre for mathematics in Toulouse, France, which analyses meteorological data, are leading a three-year project in massively parallel processing as part of an ESPRIT programme to promote information technology in Europe.

Funds of ECU15 million (US\$21.8 million) will be used to provide hardware and develop software for a technology that competes with standard vector computers. Each laboratory, which is obliged to match the grant from the European Commission (EC) with money from its own budget, will be installing a scalable CS-2 parallel computing machine built by the British company Meiko. The computers will be used to develop software to analyse vast amounts of particle physics and meteorological data, which are currently analysed by Cray vector supercomputers.

Parallel processing is capable of higher overall performance compared to vector computers, particularly important for specialist scientific, high-input high-output data analysis such as that generated at CERN. "We are not a typical vector user", says Fabrizio Gagliari, project manager at CERN, "because our work is as much computer intensive as data intensive". Massively parallel computers have the added advantage, for all users, of being much cheaper to build and to run than vectors.

The EC hopes that the project, which began last month, will increase user confidence in the potential of the European massively parallel computers and will create a market for supporting companies. A recent report on high-performance computing in Europe, compiled by CERN director Carlo Rubbia for the EC, concludes that Europe can compete effectively with US companies, traditionally the leaders in the supercomputing field, provided that support is given to overcome the perennial problem of turning basic research results into competitive products. The EC hopes to build upon Europe's acknowledged strength in software development.

Japan, although further behind in the race, is forming collaborations to close the gap. Last month NEC signed an agreement with the Swiss scientific computing centre (CSCS) to develop applications for massively parallel computers in a new, NEC-funded high performance computing software development centre on the CSCS site.

Meiko is part of a three-way consortium of suppliers that will benefit from the EC project; its partners are the British company Parsys and the French company Telnat.

Alison Abbott

New, greener ethos bolsters bill to protect California desert

Washington. A 17-year effort to protect some seven million acres of federally owned desert in California seems to be on the brink of success, the result of a new president and changing attitudes in Congress.

A campaign by environmental groups to shield the fragile Mojave and Sonoran deserts from the impact of mining, ranching, military and off-road use has long been opposed by the Reagan and Bush administrations and by the lobbying interests of the people who work and play in the desert. But Interior Secretary Bruce Babbitt has pledged that preserving California's desert will be the first land conservation goal of the Clinton administration. Babbitt's announcement came a month after California's two newly elected Democratic senators introduced a bill to preserve one-third of the federally owned desert land in their state, a measure that is expected to become law by this summer.

The Mojave and Sonoran deserts cover 40,000 square miles of remarkably diverse terrain. Sand dunes, dry lakes, extinct volcanoes and 90 mountain ranges dot the landscape, and there are 760 animal and 1,300 plant species.

The California Desert Protection Act would create a 1.5 million-acre Mojave National Monument in the East Mojave, which houses the world's largest Joshua tree groves as well as the greatest population of the threatened desert tortoise. It would transfer supervision of the areas from the Bureau of Land Management (BLM) to the National Parks Service and would bring to an end the existing cattle ranching, mining activity and off-road vehicle use. The bill would also add 1.3 million acres to Death Valley and 200,000 acres to Joshua Tree national monuments, transforming both into more tightly controlled national parks.

"We've all seen this initiative stymied far too long", Senator Dianne Feinstein said about her bill, cosponsored by Senator Barbara Boxer. "Now the important thing is to get the ball rolling and protect what's left of the desert."

The federal government owns about half of California's entire desert wilderness. Of that land, about 15 per cent is used by the military as training grounds for flight, bombing and land warfare exercises. On any given day, sonic booms rattle the land near Edwards Air Force Base, while soldiers practising tank battles at Fort Irwin leave dust clouds visible more than a hundred miles away.

The most controversial aspect of the

proposed legislation would set aside an additional four million acres of desert as "federal wilderness areas", prohibiting roads and motor vehicles and limiting mining, military activity and livestock grazing. It is not surprising, therefore, that most people who earn their livelihood in the desert oppose the measure.

"It's going to kill us", says Dave Fisher, who for 29 years has operated two ranches totalling 270,000 acres in the western

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The desert tortoise is one of many species in the protected area.

Mojave; some 80 per cent would be reserved as wilderness under the legislation. The bill's effect on mining, the desert's biggest industry, would be even more dramatic. The ending of mining rights would lead to a loss of some 20,000 jobs and \$2 billion in revenue over the next seven years.

"If concessions aren't made for those who live in the desert, the results would be economically disastrous", says US Representative Jerry Lewis (Republican, California), who represents most of the rural Mojave.

Most of the federally owned land in the area is run by BLM, which has apportioned it for extensive commercial mining and ranching as well as for recreational use. Each year, mining levels mountain peaks and kills off plant and animal species that have adapted to the habitat over thousands of years. Equally threatening are the dozens of federally subsidized cattle ranches that have depleted underground water sources used by antelope and bighorn sheep. The BLM has also set aside 1.5 million acres of formerly pristine wilderness for unlimited shooting ranges and for use by jeeps, dune buggies and dirt bikes.

More recently, the BLM has leased large tracts of wilderness to be used as garbage dumps for cities in the Los Angeles area. And later this year, the agency plans to sell land near the Colorado River for the nation's largest unlined low-level radioactive waste dump.

Susan Greene

Cost overruns, management problems found in British Antarctic projects

London. The British Antarctic Survey (BAS) received a public rebuke last week from the government's financial watchdog for its handling of several capital projects during the past few years.

The National Audit Office (NAO) complimented BAS on three of four projects and on the general quality of its research. But it criticized some of the procedures used, including those for the construction of a new research and supply vessel, the *RRS James Clark Ross*, and for an airstrip and berthing jetty at its Rothera station. The total cost has been several million pounds more than budgeted.

The budget of BAS, which is part of the Natural Environment Research Council, rose nearly fourfold, to £52.4 million (US\$75 million) in 1990–91, in the wake of the 1982 Falklands War following a decision by the then Prime Minister Margaret Thatcher to raise Britain's profile in the region. As a result, Antarctic science was one of the few research disciplines to receive a significant funding boost in the 1980s.

A large proportion of the new money has been spent to improve access for scientists. In addition to building the *RRS James Clark Ross* and the Rothera airstrip, BAS replaced its research station Halley, located on a fast moving ice-shelf, and bought a De Havilland Dash 7 aircraft, which was converted for Antarctic operation.

There were cost overruns on each project and also, in the last two cases, delays in completion. Design specifications for the research ship, for example, were still being finalized even after the design contract had been signed. Originally estimated to cost £30 million, the ship was budgeted at £40.8 million and wound up costing £42.7 million.

Similarly, inadequate information about the siting of the jetty at Rothera meant that it had to be redesigned and repositioned at a relatively late stage, at an additional cost of £2 million. BAS spent £9.4 million, £3.4 million more than planned, to rebuild Halley, and the project took two years longer than expected. In each case, according to the audit office, BAS devoted insufficient resources to project management and failed to train and support the project managers adequately. The project officer for the *RRS James Clark Ross*, for example, lacked knowledge of ship construction and experience with large projects.

BAS director David Drewry is withholding detailed comments on the criticisms until after parliament's public accounts

committee has discussed the NAO's report next month. But he believes that "the report clearly indicates that, as far as taxpayers are concerned — and given the difficulties of operating in one of the most

overruns have had little overall impact on the scientific programmes. However, he says that the points raised in the report have been "taken seriously" by BAS, whose experience should help other groups faced with similar challenges.

Meanwhile, BAS is due to announce this week how it intends to spend an extra £4 million awarded by the Office of Science and Technology over the next three years. A significant proportion of this sum will be used for clean-up operations required under the terms of the Antarctic Treaty signed in Madrid in 1991. Part of the money will also be used to refurbish the Signy research station.

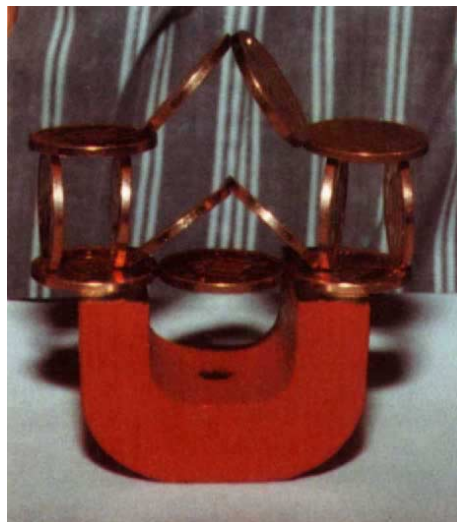
The money is considerably less than the £12 million that BAS is reported to have requested and has raised fears that some research projects may be terminated. Drewry says that BAS intends to maintain its current level of scientific activity and that there are no plans for redundancies among BAS staff.

David Dickson

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Ice was not the only obstacle to building the *RRS James Clark Ross*.

hostile parts of the planet — they have had value for money". Drewry says that the problems with contract management were "second-order issues" and that the cost



Magnetic money

London. The ever decreasing value of coinage is the common currency of taxi driver and bar talk, but a new cut-price penny with surprising magnetic properties is the sole interest of an international Magic Penny Society, founded by Robin Willson, professor of Biochemistry at Brunel University.

Last September the Royal Mint stopped making penny and two-penny coins from bronze and started instead to electroplate steel disks with copper. Willson first knew of this when one of the coins stuck to his magnetic key-retainer — the steel core is a soft magnet that becomes magnetized in an applied field. Fields minimize their energy

by funnelling through the coins, which consequently stick together.

Initially, Willson used the effect to entertain children at the leukaemia unit in St Bartholomew's hospital, but when he found that scientists also were taking interest, he founded the Magic Penny Society, partly because he sees the new pennies as an excellent educational toy to excite children's interest in science.

Already featured at the British Science Museum, the Bristol Exploratory, in Singapore (where one onlooker built the penny pagoda, above), and even with one of Faraday's original electromagnets at the Royal Institution, the clever currency will next appear at the Edinburgh Science Festival next week.

Roland Pease

Carnegie completes review of science-government ties

Washington. Participants in a five-year effort by the Carnegie Corporation to bring science and government closer together believe that they have improved that relationship somewhat and hold out the promise of even greater success. But critics say that the project failed to recognize the diversity of the US scientific community and that it is very difficult for any non-governmental group to bring about significant and lasting change.

Last week, the Carnegie Commission on Science, Technology and Government issued a summary report on the results of a \$10.7-million project begun in 1988. The

he nominated Gibbons on Christmas Eve, a month before taking office himself.

There have been other successes, according to the commission's executive director, David Robinson. The group's proposal for a joint office between the National Science Foundation and the Department of Education has been partially met by the recent signing of a formal "memorandum of understanding" between the two. Robinson also cited the work of the commission's judicial task force in recommending and helping to set up a scientific organization inside the Federal Judicial Center that will provide guidance for judges on the question of interpreting scientific evidence in court (see page 481).

Gibbons, speaking last week at the unveiling of the commission's final summary report, said he was struck by how many of those involved are now — like himself — part of the new administration. The commission's defence task force, which called for radical changes in military procurement practice, was led by Bill Perry, the new deputy secretary of defence. "The difficulties of integrating civil and military technology are even greater than envisaged", Perry said last week, "but we will persevere".

Responding to criticism that the commission had said next to nothing about the role of university scientists, Joshua Lederberg, president emeritus of the Rockefeller University in New York and co-chairman of the commission, said that any such comments would have been dismissed as special pleading. But more telling criticisms may be the restricted nature of the body from which it drew advice and the lack of any formal mechanism to follow through on its reports.

"They should get credit for the informal aspects of what they did, and their staff work was superb", says Daryl Chubin, a senior associate with the congressional Office of Technology Assessment. "But the process was pretty closed. With any organization that consists almost entirely of white males over 50, you are talking about one particular view of the world, and that worries me." Nearly half of the commission's 17 panels contained no women, for example, and fewer than half a dozen minorities participated in the exercise. Commission officials say that they sought out the most talented people in each field, regardless of gender or race.

As for follow-through, Robinson will continue to run a small office at the corporation to monitor implementation of the commission's proposals over the next two years.

Colin Macilwain

South Africa tightens spending for universities

Cape Town. The South African budget for this year gives university researchers less than they need to keep pace with inflation and allocates most of the additional spending on science to applied work in government laboratories, dashing hopes of a significant increase in university funding. Not only does the increase of 6 per cent in the science budget fall short of a general increase in spending of 9 per cent and an inflation rate of 10 per cent, but four-fifths of the additional \$13 million will be allocated to the Council for Scientific and Industrial Research (CSIR), which carries out in-house research on applied science and technology.

The allocation runs counter to the recommendations of a report last year that asked for greater spending on universities (see *Nature* 356, 9; 1992). The impact of the report appears to have been confined to a reduction of 6 per cent, to \$68 million, in the allocation to the Agricultural Research Council (ARC). The two largest councils, the ARC and the CSIR, together get 61 per cent of the science budget.

Friedel Sellschop, deputy vice-chancellor (research) at the University of the Witwatersrand, says that the share of the budget going to universities "remains dangerously inadequate by international standards". Jennifer Thomson, a former director of the CSIR's Laboratory for Molecular and Cell Biology now at the University of Cape Town, interprets the allocation as an attempt by the government to rescue the CSIR in times of recession and a continuing decline in military contracts. Since it was restructured five years ago (see *Nature* 332, 197; 1988), the CSIR has tried to obtain a larger proportion of its income from both the private and public sectors for consulting. In an article co-authored by Johan Lutjeharms, Thomson writes in the current issue of *South African Journal of Science* that "it would probably be true to say that science as a thriving, innovative, intellectual enterprise within the CSIR is dead".

The reduction in the ARC's subsidy is a belated acknowledgement that agricultural research is overfunded (see *Nature* 362, 386; 1993). The allocations to the other councils are:

- CSIR, \$75 million (up 16 per cent);
- Human Sciences Research Council, \$24 million (up 6 per cent);
- Medical Research Council, \$14 million (up 7 per cent);
- Council for Mineral Technology, \$18 million (up 7 per cent);
- Foundation for Research Development, \$37 million (up 11 per cent).

Michael Cherry

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Joshua Lederberg (left) and William Golden

commission drew on the knowledge of some 160 senior figures in science and public policy, including former US presidents Jimmy Carter and Gerald Ford, and distributed 208,000 copies of 19 separate reports.

William Golden, co-chair of the commission, says that the group's work will attract "more recognition in the twenty-first century" than it does today. Even so, he says, the commission can claim credit for several short-term victories, including a larger role for the president's science adviser and improved liaison between some government departments and related federal agencies.

Critics say, however, that the commission failed to obtain the views of those outside the academic and political establishment and that any progress has come about independently of its work. Golden, whose science policy roots go back to the presidency of Harry Truman, retorts: "Paternity is always hard to prove".

The commission's first report, *Science, Technology and the President*, was issued shortly before the election of President George Bush. It called for the appointment of a science adviser at cabinet level and early enough in the new president's term to help him fill other scientific posts, an expanded Office of Science and Technology Policy (OSTP) and access to expert outside advice. Bush fulfilled all those requests except for promptness; that was met by Clinton when

US court gives original owners the right to deep-sea treasure

Washington. High-technology adventurers may think twice before seeking sunken treasure after the US Supreme Court let stand a ruling that insurance companies have a claim on the booty. The possibility of extended legal battles, salvagers say, may stifle the technological and scientific progress that accompanies the recovery of shipwrecks.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Gold coins and bars lie amid soft coral stalks covering timbers from the SS *Central America*.

The supreme court declined to hear a case involving a ton of gold recovered so far from the SS *Central America*, resting in 8,000 feet of water off the coast of South Carolina after being sunk by a hurricane in 1857. Their action let stand a decision by the Fourth Circuit Court of Appeals in Richmond, Virginia, that property lost at sea can be abandoned only through the failure of insurers to appear in court or through a written statement relinquishing title.

Several groups have tried to locate the wreck in the past 20 years since technology made possible the recovery of its contents. They discussed purchasing the rights to the treasure with the insurance companies, but no contracts were signed.

The district court originally gave the gold to the successful salvage team, the Columbus-America Discovery Group of Columbus, Ohio, by applying a finders-keepers rule known as the law of finds. It said that the Atlantic Mutual Insurance Company and eight other insurers had abandoned their stake by destroying or losing all records of paying the claims.

The appellate court instructed the district court to apply the law of salvage instead, determining the share of salvagers and insurers according to the cost of recovery and the care taken by the salvagers to preserve the historical and archaeological value of the wreck. As an incentive to preserving wrecks, court awards to conscientious salvagers often approach the total value of the treasure, according to Porter Hoagland, an expert on marine policy for the Woods Hole (Massachusetts) Oceanographic Institute.

But the lawyer for Columbus-America, Richard Robol, says that the law of salvage undermines the financial incentives to develop the necessary technology for a high-risk venture that requires large amounts of capital. Columbus-America investors spent

more than \$12 million, in large part to develop a sophisticated unmanned submersible, called Nemo, for a return that may be worth as much as \$1 billion.

Columbus-America designed Nemo to remain at depths of 1.5 miles for several days. In addition, the group developed robotic equipment, using three-dimensional video monitors, that is sensitive enough to pick up single gold coins and to lift heavy beams.

Biologists have studied the nutrient-rich environment created from the wood and iron in the shipwreck in an otherwise barren area by using hundreds of hours of tape from Nemo's cameras. "Having three-dimensional viewing has almost taken away my desire to go down there myself", says Bob Evans, director of the group's science projects. The new equipment has also enabled them to recover biological samples, including a crab holding eggs tucked under its tail and sponges that are being tested for medically active chemicals.

Jenna Roberts

Catalonia plans for synchrotron

Barcelona. The autonomous region of Catalonia has decided to build a synchrotron facility near Barcelona following rejection last December by CERN, the international particle physics laboratory in Geneva, of a request by Spain for support of a facility to produce large numbers of tau and charm particles.

Pure and applied research in microelectronics, biochemistry and material sciences, areas of particular economic importance to Catalonia, will be conducted by a large permanent staff as well as by visiting scientists from the rest of Spain, Portugal and southern France. The synchrotron should also offer an entry point into Europe for scientists from Latin America.

The Spanish government has promised to pay some of the Pts11 billion (US\$95 million) cost of building the Barcelona synchrotron, and the European Communities have been asked to provide half of the money. But Catalonia says that it will bear the entire cost if necessary.

An international advisory group headed by Catalan physicist Manuel Cardona, a director at the Max Planck Institute for Solid State Research in Stuttgart, Germany, is developing specifications. The 2.5 GeV synchrotron, which will have a perimeter of 250 metres and 20 workstations, is to be built 20 km from Barcelona. The site is near the Autonomous University of Barcelona and the Vallès Technological Park and close to two large centres for microelectronics and material sciences. Construction is expected to begin next year and the first experiments could be under way as early as 1998.

Xavier Duran

Genome project 'to be done by 1994'

Washington. The human genome project, which aims to identify the make-up of all human genes, could be completed in a purpose-built US laboratory by the middle of next year, according to a leading participant in the work.

Craig Venter, the former NIH official who set up the \$70 million Institute for Genomic Research in Maryland specifically to pursue the project, told a *Nature Genetics* conference in Washington last week that he hopes to have "a nearly complete description of the human genome, in the freezer and on computer, within twelve to eighteen months".

Mark Adams, head of gene sequencing at the institute, said that the latest estimate compared with a three- to five-year estimate when work started last summer, thanks partly to more resources being

devoted to the project and partly to better methods for finding new genes quickly and without redundant effort.

Venter says that his facility, which employs some 50 robotic DNA sequencers, is finding between 500 and 1,000 new gene sequences a day, including partial sequences. He estimates that the project is already 20–25 per cent complete, and that the total number of human genes may be around 75,000. "We'll know the number for sure in a few months as we get closer to completion", he says.

The institute is responding to concern about the wider social and medical consequences of the project by establishing an ethics division, and by releasing groups of its gene sequences for general research use.

Colin Macilwain

Greek university law defended

SIR — I wish to comment on the letter from Emmanuel Papamichael (*Nature* 360, 704; 1992) on the new university law recently adopted by the Greek parliament. The letter is one-sided and misleading.

Before the law was introduced in the Greek parliament, there was an extremely long procedure during which the minister of education visited each Greek campus and held lengthy discussions with the universities' senates on the basis of written replies to a questionnaire distributed by the ministry to each member of the university community in Greece. It is therefore misleading to suggest that there has been "barely any debate".

Either by accident or design, Papamichael has also failed to mention several of the chief features of the new law. Thus it includes provisions for allowing the emergence of new institutions, one of which is the new open university aimed at providing extramural education for particular groups in Greek society and the foundation of a centre for the Greek language to coordinate the maintenance and dissemination of Greek in the European environment. Similarly, the law has made possible regulations to encourage the mobility of Greek university students within Europe. The arrangements for the accreditation of Greek universities is a new procedure aimed at rationalizing the distribution of funds according to objective criteria.

The new law also provides a simple and straightforward legal framework for the foundation of postgraduate courses, which have so far been weak and virtually nonexistent. Universities can now apply for funds to found postgraduate departments as well as to create academic research institutes linking universities with the industrial and business world.

At the same time, the new law provides that the previously free distribution of textbooks to all students be confined to needy students only. The funds set aside in this way will be used exclusively for departmental and central university libraries. Papamichael might have also mentioned that the number of scholarships and the funds available for them have been increased by 50 per cent and 500 per cent respectively.

Papamichael is also concerned with the status of existing academic personnel. But neither the old nor the new law provides tenure for the two lower academic grades. Papamichael may be right in saying that in Greece there is "very little employment in the private sector", but he fails to mention that only under the new law people who fail to get

tenure at universities are now eligible for employment in the public sector. Moreover, it is not true that under the new law there is a freeze on promotions. Rather, the new law requires that academic positions be filled by responses to open advertisements and that the disciplines concerned be determined by the universities and not by the ministry. The ministry's power consists only of agreeing the number of places to be advertised, for which of course it has to pay the bill.

Papamichael's hope that the universities would have been overtaken by protests and that Greek scholars would have boycotted the examinations is wishful thinking. The universities are working normally under the new law.

Papamichael's account of the "private universities" is also inaccurate, as they are illegal under the Greek constitution. His comments on the absence of the minister from European meetings leaves no doubt of his one-sided and political approach to the problems of higher education in Greece.

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Copyright scam

SIR — I am concerned at the cavalier way in which the copyrights of scientific papers are handed over to commercial publishers.

Increasingly the status and pay of scientists are determined by what they publish and by ratings in the *Science Citation Index*. Indeed they have to publish or perish ("Sure he was a great teacher, but what did he publish?") But in what other profession are the products of one's labour given away for nothing to people who then sell them?

I have been making a collection of the agreements that scientific authors are required to sign to get their papers published. I find some of them quite horrifying. Not only copyright, but all rights, are often signed away.

Twice recently, papers submitted to a journal have later appeared as sections of a book. The situation appears to be very different from that in the literary world, where, for example, copyrights of minor works by T. S. Eliot are objects of immense value. Copyright in chapters for a scientific textbook are often bought for sums that would hardly cover the cost of typing.

Dangerous conditions are sometimes attached. One publisher requires that

authors warrant that the article does not infringe any copyright, trademark or patent, that it is not libellous and so on (matters that are beyond the competence of the author to judge) and the author is required to indemnify the publishers "against any costs, expenses, or damages which [the publisher] may incur or for which [the publisher] may become liable as a result of any breach of these warranties". The publisher has formed a limited liability company to protect himself from such contingencies but nevertheless has tried to pass this liability to the private individual. Whether an item is libellous or whatever depends on where it is circulated and this is under the control of the publisher and not of the author.

I think that authors requiring protection should assign copyrights to their institutions, or to the research council supporting the work, and inform the publisher that this body will give the publisher an exclusive licence to the material for perhaps two years. I have tried this a couple of times and have got away with it.

Publishers have to make an exception for work produced by US government employees, where copyright is not transferred to the publishers, and this causes, I believe, no trouble at all.

With increasing commercialism, research councils and universities have a duty, I consider, to help to protect their authors in this matter.

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Tobacco code

SIR — Because direct advertising of cigarettes is forbidden in Italy, it seems that other insidious strategies for cigarette promotion are being developed. But to my knowledge, never in the past has a Nobel prizewinner in medicine signed a promotion for Philip Morris Companies Inc. This year, however, new year greetings from Philip Morris, specifically addressed to the younger generation, occupied a whole page of the Italian newspaper *La Repubblica* on 31 December 1992, and included a message signed by the Nobel prizewinner Dr Rita Levi-Montalcini under the heading "Culture of the modern time". As a physician of Italian origin, I can only express a sense of surprise and shame which, I hope, is shared by the whole Italian biomedical community.

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Opinion and fact and UK energy

SIR — John Surrey and Mike Parker have firmly held opinions on nuclear power (*Nature* 361, 677–680; 1993), but they should not be construed as facts.

Opinion: “New nuclear stations were uneconomic when the discount rate was raised to 8% in 1988 . . . The breakthrough with nuclear capital costs has not been achieved . . .”

Fact: Sizewell B is ahead of schedule, within the £2.03 billion budget which includes all first-of-a-kind costs, will be profitable and will contribute significantly to Nuclear Electric's future cash flow. A twin pressurized water reactor (PWR) Sizewell C, replicating the design, has been costed in detail. It could be built, operated for 40 years, fully decommissioned — and still produce at well under 3 pence per unit at an 8 per cent rate of return, competitive with new coal, new gas and French nuclear stations. The design is exciting international interest; Westinghouse has asked Nuclear Electric to join it in a joint bid for Taiwan's new nuclear station on precisely this design.

Opinion: “. . . it will not be known until Sizewell B has been operating for some years whether the PWR will become the established nuclear option that has been sought for 30 years.”

Fact: With more than 150 operating worldwide over 30 years, the PWR already is the established international option. The Sizewell B design is based on the Westinghouse reactors operating for the past eight years at Wolf Creek and Callaway in the United States. Both stations routinely appear at the top of the performance league, with load factors well in excess of 80 per cent. Sizewell B, with enhanced diversity in control and monitoring systems, will certainly produce at over 85 per cent load factor.

Opinion: “The total liability for nuclear decommissioning costs now stands at £18–£20 billion . . . £5.4 billion for the Magnox stations and £4.2 billion for the AGRs . . .”

Fact: The authors have chosen here to quote undiscounted and out-of-date figures although they make much play of discount rates elsewhere in their article and must be aware that the decommissioning costs concerned arise over a very long period of time stretching to the end of the twenty-first century and beyond. As our evidence to the Select Committee showed, we have made full provision for these costs in our accounts. Their present value is £2.5 billion using a very conservative discount rate of 2 per cent. The Select Committee suggested that these costs might actually be too high and it is true that if our ‘Safestore’ strategy is adopted we will be able to

reduce the liability by at least a further £500 million. It is completely irrelevant to quote the decommissioning costs associated with Ministry of Defence activities in what purports to be an article about civil nuclear power.

Opinion: “Nuclear objectives proved inherently more difficult to achieve . . .”

Fact: Yes — until 1989. Since then, Nuclear Electric is achieving them. In less than three years, output is up 25 per cent to record levels, productivity is up 50 per cent, unit costs are down by almost a third, and Sizewell B is ahead of schedule and within budget.

Opinion: “The levy . . . which finances more than half of nuclear generating costs.”

Fact: No it doesn't. Nuclear Electric inherited liabilities from CEBG operations of £10.5 billion, without any of the provisions to cover them. The levy, which reduces in real terms year on year, will raise a total of £9.1 billion. However you do the sums, the levy is clearly there to pay for history; the Select Committee said it is “indefensible that so much of the burden of discharging the pre-April 1990 liabilities should be placed on electricity consumers within one eight-year period”. But to suggest it's an inevitable part of our continuing generation costs is simply wrong. It has nothing to do with the commercial future of nuclear power.

Still, there's one overriding opinion on which we can all agree. As Surrey and Parker put it: “Whatever decisions flow from the government's policy review, it is important to understand the background . . . Throughout, there was no formal framework of energy policy.”

Let's get it right this time.

John G. Collier

(Chairman)

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Patent problems

SIR — Your leading article (*Nature* 361, 192; 1993) on the patenting of transgenic plants illustrates some of the complexities being faced by the European Patent Office from applications to patent transgenic agricultural plants and animals. You highlight the problems likely to become apparent should animal breeds or plant varieties be patented; there would be established a double system of monopoly rights and subsequent confusion over ownership. No doubt lawyers would be happy with this but conventional breeding programmes could well suffer.

Article 53 (b) of the European Patent

Convention (EPC) currently excludes plant varieties from patentability. The response of appellant patentee lawyers has been to claim exclusive rights to ‘plants’ which will, in fact, include all varieties of plants containing the gene in question. Besides being a linguistic contortion to avoid the use of the phrase ‘plant variety’, this all-embracing course of action seems merely to exacerbate the problems.

Of equal significance to biologists is another part of Article 53 (b) which prohibits the patenting of ‘essentially biological processes’. This was included, no doubt, because those who drafted the convention wished to avoid the patenting of living matter, although an exception was made for microbiological processes. Applicants for patents on transgenic organisms are now arguing that where the inserted genes come from microbiological sources, the organisms being patented have suddenly become microbiological. Second, they argue that because human intervention is involved in the production of transgenic plants or animals, they have somehow ceased to be biological and thus patentable. Such assertions run contrary to the organizational and integrative principles of biology. It is crucial that more biologists now join this debate on the patenting of life forms to forestall the conveyance of their subject into a legalistic and linguistic Wonderland.

A patent is also meant to be granted in the public interest and the exclusion clause concerning public order and morality is to ensure that patents fulfil this obligation. The granting of patents on plants rewards a technology that could lead to the genetic pollution of nature, an outcome that is clearly not in the public interest. The wording of the EPC, however, is difficult to apply to morality issues surrounding genetically engineered organisms for exactly the same reasons that difficulties arise over ‘essentially biological processes’ — the drafters of the convention did not envisage patenting of plants and animals.

The European Patent Office has got itself into a mess over these issues because it has proceeded to patent life forms without first examining properly whether the EPC, when interpreted as it was originally intended, actually allows the patents the patent office has granted. It has allowed itself to be pushed into juggling words to fit industries' interests and has ignored the legitimate moral concerns of many. What it should do is to stop issuing patents for plants and animals and await the outcome of proper international debate.

Susan Mayer

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The shadow of genetic injustice

Benno Müller-Hill

The effects of the inevitable discoveries emerging from the Human Genome Project will be catastrophic for some. Now is the time for preventative action to be taken.

MANY knowledgeable people have a deep dislike of the Human Genome Project. If you ask them why, some may say that the money would be better spent on different, smaller projects. A few fear that the outcome could be harmful; they foresee a new underclass, unable to get jobs or insurance. These pessimists of course overlook that this underclass already exists: 37 million people have no health insurance of any kind in the United States. Could things become worse?

Proponents of the Human Genome Project imagine great progress in diagnosis of diseases and in their therapy. Some predict that eventually everyone will carry all the sequences of his genes on a compact disk. Others even claim that the present knowledge of man and his mind is nil and that real culture will arise only out of the knowledge of the sequence of human DNA. This, I think, is unlikely.

As pure scientists, these enthusiasts often do not imagine the consequences for society as a whole. My own view is that the project will be truly enlightening about the molecular structure of the human body and brain, and that this knowledge alone is exciting enough to justify the programme. Little is known in molecular biology and many unsuspected medical applications may emerge from this endeavour. As a molecular geneticist, I have always defended the Human Genome Project as scientifically sound and important, and I continue to do so. But I have also tried to spread the knowledge of the past criminal misuse of human genetics in Nazi Germany. Until now I have abstained from commenting on the possible effects of the Human Genome Project on society in the future. But science after all consists of making, and testing, predictions. So I have de-

cided to stick my neck out and to make some predictions for the next 30 years.

There is no doubt that the main medical progress will be in diagnostics. Therapeutics may lag behind, possibly for decades, so I will abstain from discussing these aspects here. What will be the main diagnostic results of the Human Genome Project that are relevant to society? I think there are two different areas: genetic ailments of the body and those of the mind.

There are many severe ailments of the body for which there are genetic predispositions. Most of these conditions are extremely rare: only a very few are as frequent as one per cent. At the moment it is in most cases just about possible to say that, according to a family history, a certain risk exists. The Human Genome Project, however, will lead to the identification of DNA defects in the relevant genes. Then, accurate predictions can be made from the blood (DNA): for example, one will be able to predict that a healthy child will die in his or her forties from Huntington's chorea or from familial Alzheimer's disease. In Huntington's chorea, the time-point of the outbreak of the first symptoms will vary considerably. In other diseases it may vary even more. The predictions will thus not be accurate in the sense that the severeness of the symptoms can be predicted for a particular time, but they will be statistically accurate. This concept is difficult to explain to patients and clients.

There are some similarities between this situation and that in the late nineteenth century, when infections were random and where no treatment was available. Then, many had the bad luck to be infected whereas here we are talking about only a few people. But the late nineteenth century was the time that

general health insurance was introduced in countries like Germany. Bismarck's reform in that country had the effect of increasing the industrial output of Germany. Perhaps the time of molecular revelation of rare diseases would be the proper time to introduce general health insurance in the United States?

To continue my predictions for the future. Imagine four potential cases of genetic disablement. (1) When a haemophilic wants to become a butcher it makes sense to discourage him. (2) When a colour-blind man wants to become a truck driver, he fails at a colour test: his DNA does not need to be tested. (3) When a healthy young man asks for employment and his employer wants to know whether his genotype indicates that he may die in his thirties or forties from Huntington's disease, this is unfair. (4) When a healthy black person seeks employment and is tested for the sickle-cell anaemia gene, then is refused employment because there may be an extra risk under certain conditions, this is unfair.

Thus, in my view, practical ability tests are acceptable, but genetic tests for disabilities which may turn up later in life should not be made available to employers. Moreover, DNA testing for employment purposes should not be performed if a large ethnic group would suffer. But it goes without saying that a person presenting a potential employer or insurance company with unsolicited evidence that he does not carry the genes predisposing for various ailments will have a definite advantage.

One can imagine the development of protest movements among those who have decided not to reveal their genetic identity, and there will be fundamentalists who will never have themselves tested because they themselves do not want to know. All these groups will suffer grave social disadvantages. One can also imagine clubs for the "healthy" that will demand proof of "genetic fitness". The turning point may come when the US supreme court decides that genetic injustice is so immense that it is within the law to show an employer or the insurance falsified genetic identity. (In Germany the supreme court has just decided that pregnant women may lie about the pregnancy to an employer.) From then on, the interest of employers and insurance companies in the genetic



Predictive powers — a DNA sequence of the HL-A gene cluster from chromosome 6, being analysed as part of the Human Genome Project.

identity of their employees or clients will wane.

I think all these developments will be painful but could come to a good end: the genetic bad luck of a very few may, eventually, be helped by many in that general health insurance and tough anti-discrimination laws could become reality. It is, of course, also possible that the genetically handicapped will be simply too few ever to influence the majority to come to their help.

The situation will be different with the genetic ailments of the mind, which I shall now discuss. Here, the percentage of the population involved is much larger and cannot easily be overlooked. Any current textbook of human genetics will contain the assertion that most differences of the human mind and soul (to use an old-fashioned word) are inherited. But in almost all these cases the respective genes have not been located or isolated, and the DNA defects are thus totally unknown. It is claimed that psychiatric diseases such as manic depression or schizophrenia, and also psychological disablements such as low intelligence, are somehow inherited. But the truth is that the extent of family influence or other environmental factors is unclear. Proof that these ailments are essentially genetically determined can come only through the knowledge of the DNA sequences of these genes and their variants. Knowledge of the DNA sequences of a person will then allow one to make statistically accurate predictions about that person's intelligence and mental stability, and thus about their possible fate.

In earlier times, such predictions were the business of charlatans. But belief increases the likelihood of a predicted outcome: placebos against psychic ailments work astonishingly well. The scientific prediction of a person's limitations, and thus his possible fate, has a very dangerous component in that it may lead the individual to inaction and despair. It may also lead the population at large to believe that, as there is no real chance, money should not be wasted to counteract genetic limitations. It could be forgotten that these limitations are also set by environmental factors.

Many medical scientists would like the fame of having isolated one of these genes. Some succumb to the pressure and claim they have obtained evidence where in fact there is little or none. The 'discovery' of genes determining alcoholism, schizophrenia, manic depression and Alzheimer's disease have been peer-reviewed and published in leading scientific journals. They were hailed in the media as breakthroughs — and then they were shown to be wrong either by their own authors or by others. These errors do not mean that the main qualities of

the mind and the soul are not biologically inherited: they show simply that scientists are ordinary people. One can predict safely that many more such non-discoveries will be made. This will be confusing for scientists and painful for the particular patients involved. But eventually the truth will emerge in areas where now we can only make guesses.

Finally, I would like to imagine what may happen if it does turn out that mental health can be shown to have a genetic basis. Let us assume as an example that a gene is isolated which in several mutated forms predisposes for schizophrenia. It will take by then a matter of weeks to determine the complete DNA sequence of such a gene. Will the sequence reveal anything about schizophrenia? For a molecular biologist, knowledge of a sequence means that the function of the protein specified by that sequence is known — for example the protein may form an ion channel or be an enzyme metabolizing a particular molecule. Does this mean that we will understand schizophrenia if we know that it often occurs in people when a certain ion channel or a certain enzyme is damaged?

Many scientists or doctors would answer in the affirmative, but I would like to say, emphatically, that the answer is no. Understanding a biochemical defect brings us no nearer to understanding the thoughts and actions of the schizophrenic. Those opposed to my view would argue that knowledge of the gene will allow us to identify which lifestyles are dangerous for such a person. But we do not need any knowledge of the gene or its product to do this. Pharmacologists, on the other hand, will point out that they now can design rational drugs which may influence precisely the functioning of the relevant gene product. But it is not true that if we know which drug to prescribe, we know all that is necessary to know about schizophrenia. Although the molecular-genetic approach will certainly lead to a frenzy of new drugs on the market, in the end the suffering patients will be helped only partially. It is so much easier to prescribe a pill than to change the social conditions that may be responsible for the severity of the symptoms.

I have little faith in the notion that treatment of mental diseases will truly benefit from knowledge of the culprit genes and gene products. But I have no doubt that diagnosis will flourish. Cheap tests will be developed which will allow everyone to be tested for the variants of genes determining psychiatric ailments or psychic qualities outside the doctor's office. Those carrying the disabling genes of schizophrenia, manic depression and low intelligence may constitute ten or more per cent of the US or

European population. Medical scientists will test anonymously all possible ethnic and social groups for these genes, and doubtless some ethnic differences will be found. Suddenly, genetic racial injustice will be a fact.

The isolation of the first gene involved in determining 'intelligence' (whatever that is) will be a turning point in human history. Will governments endorse the view of the eugenicists of the 1930s that the carriers of such genes are "bad and inferior" and that they and their followers are "good and superior"? Or will they stress privacy and cleverly leave the necessary selection to market forces? Or will they resort to legal measures to speed up the process of the "physical disappearance of the unwanted"? And what will the geneticists themselves say? Perhaps they will simply be relieved to find that their own mental genotypes are 'healthy'. But will some of them propose ways to eliminate the 'bad' genes from others?

Anticipating such conflicts, many may conclude that we do not need or want this genetic knowledge. I disagree. The knowledge will simply unveil reality, emphasizing the injustice of the world. It is certainly not enough to face reality bluntly if the future develops as I describe it. It is not enough simply that the right of privacy is acknowledged. If those who have this right have no education, health insurance or jobs, the right is not enough. Laws are necessary to protect the genetically disadvantaged. Social justice has to recompense genetic injustice. The details of such legislation can be spelled out only when the genetic facts are known. Deep changes in attitudes are also required. All we can do now is to be prepared. Progress may be painfully fast or slow, and will be full of contradictions. To master it demands firm values. At the extremes, people will have to choose between the values of the Nazis and those of Moses — that is, racism or an appreciation of equal human rights. The choice for politicians of the world's governments will be between international fascism or, if science and justice are combined, a fundamentally improved social structure throughout the world.

I would like to conclude by citing a sentence from *Science and the Future*, published in 1924, written by the optimist J. B. S. Haldane. "I think that the tendency of applied science is to magnify injustices until they become too intolerable to be borne, and the average man whom all the prophets and poets could not move, turns at last and extinguishes the evil at its source". □

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The next step in AIDS treatment

The apparently well-founded discovery that AZT is not a useful drug for the treatment of people infected with HIV does not mean that it is useless in the treatment of those with AIDS.

THE drug AZT, an inhibitor of DNA synthesis, has been for several years the only treatment for those with symptoms of overt AIDS. Nobody has claimed that it can clear the infectious organism, HIV, from a person's system. Rather, it has been used as a retardant of the progression of AIDS, largely on the basis that it seems to sustain the subpopulation of T cells that appears especially vulnerable to HIV in the overt stage of the disease.

Later, beginning in the United States, AZT was also used in people infected with HIV but not yet showing symptoms of overt disease, again on the basis that administration of the drug appeared to abate the decline of vulnerable T-cell counts. But now a paper in *The Lancet* (341, 889; 1993) appears to some to have thrown a spanner in the works: certainly it has caused a furore of misinterpretation by newspapers and others.

The new development is a preliminary account of an Anglo-French controlled trial of the use of AZT (proprietary name Zidovudine) in the treatment of symptomless people. It is a substantial study, including more than 1,700 people infected with HIV and divided into matching placebo and treatment groups. Although the CD4 counts of the 877 people given AZT were consistently greater than those of patients receiving only placebo, the first three years of follow-up have shown that the proportions of people in the two groups progressing to overt AIDS or even to death were not significantly different at roughly 18 per cent. The conclusions are that AZT is not an effective prophylactic of AIDS in those infected with HIV and that the CD4 cell count may not be a reliable proxy for the progression to AIDS in infected people.

Of the two conclusions, the second is the more soundly based. The first, that AZT may be ineffective in postponing the development of overt AIDS, would be more persuasive if the length of the follow-up reported in this preliminary study had been greater. It is, for example, possible that those in the treated group who have succumbed are a particularly susceptible group within the whole; whether that is likely will be more easily assessed when the data so far gathered by the study have been analysed to show the fate of subgroups, for example those with different CD4 cell counts at their recruitment. The new data are, in any case, consistent with the conclusion of the Veterans' Affairs Cooperative Study, published a year ago (*New Engl. J. Med.* 326, 437; 1992). In a comparison of the administration of AZT to infected people before and after the appearance of AIDS, early treatment was found not to affect the eventual

outcome, but merely to postpone the rapid decline of CD4 cells in the blood.

The implications of these findings are not, of course, encouraging. On the face of things, the use of AZT as a prophylactic of the emergence of AIDS in infected people is ineffective; probably it will now be abandoned. But nothing is implied by the new study of the utility of AZT in the treatment of those in whom symptoms have already appeared; there is no case for abandoning that treatment, at least on the evidence now available. It is much more disconcerting that the CD4 count has been shown to be an unreliable indicator of the effectiveness of drug treatment in HIV infection. That, so far, has been the standard test. How quickly can those concerned with the search for new drugs switch to examination of lymph-node biopsy material for this assessment? Two recent articles in *Nature* by Fauci *et al.* and Haase *et al.* (362, 355 & 359; 1993) showed that these organs (among others) are sites at which the virus multiplies during the often long latency period in AIDS, but more will have to be known of this process before it can be the basis of a routine assay.

The reaction to these developments has been marked by a note of hysteria, at least in the British press. Various, headlines have proclaimed a "bleak shadow over" or a "setback for" AIDS research. Those are justifiable, if only just. But another, in the *Sunday Times*, announces a page-long article under the banner "The cure that failed", goes on to boast of "How we broke the story" (in 1989) that treating AIDS patients with AZT might not be valid and then went on to complain that "doctors, en masse, have taken up [AIDS] as a crusade, at the expense of science". "Perhaps", it continues, "the worst feature of the medical and scientific professions' behaviour has been the enormous dominance of a single focus — HIV — for research, prevention and therapeutic efforts, to the exclusion of other approaches."

Three issues are raised by the nature of these reactions, one of which is the extremes to which criticism of both professions will be carried while there remains no cure for AIDS. It is an atavistic reaction, born partly of disappointment that decades of believing that infectious diseases are a danger past and partly from the underlying despair of those infected with HIV and the anger of the groups that represent them. It is also a dangerous reaction, if only because (in the United States at least) it is likely to mould programmes of AIDS research in directions that will not yield the benefits expected of them. Needless to say,

those infected with HIV are unlikely to be much comforted by intemperate comment of this kind.

There is also a regulatory issue. AZT was approved in the United States in 1987 for use after symptoms of AIDS have appeared, after a controlled trial was brought prematurely to an end when it emerged that the treated group was doing better than the placebo groups. Similarly, the use of AZT in early AIDS was quickly approved after the appearance of a report on a controlled trial in April 1990 (*New Engl. J. Med.* 322, 941; 1990). Those who now complain that these approvals were given too quickly overlook the sense of crisis which the spread of AIDS has engendered over the past decade, not to mention the eagerness of all concerned to match the pathos of those infected with HIV with anything that might be an effective medicine.

The position of the Wellcome Foundation, the chief manufacturer of AZT, is unenviable. Over the past few years, AZT has been the chief source of optimism about the company's fortunes. Just as the price of its shares rose dramatically at the outset, so it has now fallen. Yet there is no reason for anybody to believe that the company has behaved dishonourably. It is right now to insist that nothing in the Anglo-French study shows AZT to be ineffective in the late treatment of AIDS; it is to be hoped that point will be tested by a new controlled study. But the company is probably right also to say that several drugs in combination are likely to be involved in more effective drug therapy. Some of the justification for that may be the recent argument of Y.-K. Chow *et al.* *Nature* 361, 650; 18 February 1993).

The pattern of AIDS research is also likely to be profoundly shaped by the events of the past few weeks, but more importantly by the articles by Fauci and Haase and their colleagues than by the preliminary report of the Anglo-French study. The first and obvious need is to turn the knowledge that HIV is alive and all too well, from the outset of infection, into a workable assay of the progress of disease.

The general application of such an assay will probably in itself provide a better understanding of the pathogenesis of AIDS. But the way in which the lymph nodes (like some other organs of the immune system) sequester replicating HIV particles is so unexpected that it may well point to unexpected and more effective means for the treatment of AIDS. All that, of course, would be based on what the sceptics insist on calling the 'HIV hypothesis': there is no other, and thus no choice.

John Maddox

Orientation detectors in insects

T. S. Collett

INSECTS perform highly sophisticated visual tasks with a tiny brain. This implausible conjunction has often led people to suppose that insects analyse visual patterns in ways which avoid the need for heavy computation, and that the mechanisms involved differ sharply from those operating in mammals. But from the two papers on pages 539¹ and 541² of this issue it seems that, in at least one important respect, the mammalian visual cortex and the insect optic lobe operate upon visual patterns in a similar way. Insects, like mammals, extract the orientation of edges within the retinal image.

Bees readily learn to distinguish a pattern of stationary horizontal stripes from a similar pattern of vertical stripes. Srinivasan, Zhang and Rolfe¹ have asked how bees do so. One possibility is that they are blind to the spatial properties of the stimuli and that they can distinguish horizontal from vertical only because the two stripe-patterns activate different classes of motion detectors; that is, detectors of vertical motion will be stimulated best when a bee approaches or moves past horizontal stripes, whereas the signature of vertical stripes will be a discharge from detectors of horizontal motion. This possibility should be taken seriously, for a frequent assertion is that insects are better at analysing motion patterns than spatial ones. Indeed, the optic lobe contains a wealth of neurons which respond specifically to elaborate patterns of motion, such as the optic flow fields generated on a retina when an insect turns around or flies over the ground³, but there have been disappointingly few descriptions of neurons with interesting spatial properties.

Spatial cues

Srinivasan *et al.* now show conclusively that bees do parse visual scenes for the orientation of edges by means of purely spatial cues. Their evidence is of two kinds. First, bees can discriminate stripe orientation when patterns are presented in brief, 2-millisecond exposures, and so must be virtually stationary on the retina. Second, bees that are trained to approach horizontal stripes do not come to prefer vertically moving random-dot patterns over a similar pattern moving horizontally. On the other hand, they do favour a horizontal row of dots moving horizontally over a vertical row of dots moving vertically. So movement neither substitutes for spatial information nor disrupts it.

A feature of the stimuli is that the

widths of the stripes within each pattern are random and that the arrangement of stripes is changed from trial to trial. This means that the stimuli can be discriminated only by differences in their orientation and implies that insects have neural mechanisms for abstracting this parameter.

In the second paper², O'Carroll describes the discovery of a new class of neuron in the optic lobe of the dragonfly. These neurons resemble simple cells of the mammalian visual cortex in responding optimally to elongated bars in a specific orientation. Their orientation tuning is broad, with the response dropping to half of its maximum when the orientation changes by 90°. Although it is tempting to suggest that analogous neurons are involved in the orientation discrimination of bees, this temptation should be resisted — at least for the time being. For one thing, the neurons in the dragonfly require moving bars to activate them, whereas bees are able to recognize stationary patterns.

What is the anatomical basis of this orientation selectivity? D. O'Carroll (personal communication) suggests an answer which is reminiscent of that proposed by Sutherland⁴ to account for the ability of octopuses to recognize spatial patterns. An octopus can be taught to discriminate between vertical and horizontal bars, but it is unable to learn the difference between bars at +45° and at -45°. This finding has an anatomical correlate: the dendritic fields of cells in the octopus optic lobe tend to be elliptical with their major axis oriented predominantly horizontally or vertically⁵. So it seemed plausible that these elliptical fields are stimulated best by bars oriented parallel to their long axis, and that the lack of obliquely oriented cells explained the difficulty the octopus has in distinguishing between the two diagonals. But it was hard then to perform the necessary physiological experiments to test the idea.

Oriented dendritic fields are also seen in the optic lobes of insects⁶, and O'Carroll has now been able to correlate physiology and anatomy. He finds that the dendritic processes of the orientationally sensitive neurons cover an elliptical field. The major axis is parallel to the bar-orientation which elicits the greatest response, while the length of the minor axis equals the projection of the width of the visual receptive field.

What is the normal function of insect orientation detectors? One possibility is that they provide inputs for very specific visual reflexes. For instance, houseflies

fixed so that they can only roll about their long axis, orient themselves to keep edges aligned vertically in their frontal visual field⁷. Under normal conditions, therefore, they may rely on plant stems and trees to remain upright. The input for such a reflex could come from a single class of broadly tuned vertical orientation detectors.

Orientation channels

A more interesting function is that performed by orientation detectors in the primate visual cortex. Here each patch of visual space is analysed by about 18 different orientation channels. This enables the cortex to encode precisely and economically the orientation of an edge within the patch. The number of orientation channels in the dragonfly optic lobe is unknown, but work by M. V. Srinivasan, S. W. Zhang and K. Witney (personal communication) implies that bees have at least three — the minimum required for coding orientation unambiguously. When bees have been trained to approach horizontal rather than vertical stripes, they prefer the horizontal pattern over a uniform grey one and they prefer a grey stimulus over vertical stripes. Bees, thus, actively approach horizontal stripes and avoid vertical ones, suggesting that there are two or more independent channels. Unlike octopuses, they can also be trained to distinguish between +45° stripes and -45° stripes⁸, bringing the total to at least three.

It would be intriguing if orientation detectors play the same fundamental role in the pattern perception of mammals and of insects. Such similarities would not have surprised the great anatomist Cajal, who, overwhelmed by his first view of the insect optic lobe, wrote:

In comparing the visual ganglia from a bee or a dragonfly with those from a fish or an amphibian one is extremely surprised. The quality of the psychic machine does not increase with the zoological hierarchy. . . . It is as if we are attempting to equate the qualities of a great wall clock with those of a miniature watch. . . . [the insect's optic lobe] is a marvel of detail, delicacy and precision⁹. □

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Flip-flop end to last ice age

Richard G. Fairbanks

THE most celebrated finding in ice-core research is the record of atmospheric CO₂ concentration spanning the past 200,000 years. No doubt, future historians will debate the extent to which this record catalysed international interest in global warming forecasts. On page 527 of this issue, Richard Alley and co-workers report ice-core evidence of a different, but equally profound nature. From measurements of annual ice-layer thickness over the past 15,000 years, the authors find that Greenland's climate, emerging from the last ice age, twice shifted from glacial to interglacial conditions over an astonishingly quick 3–5 years.

This result apparently supports earlier claims that the Earth's climate system has several stable modes, between which it flips. At issue is whether these apparent climate shifts are driven by internal or external forces. Specifically, environmental scientists have posed the politically charged question: are the catastrophic climate changes measured in this and previous work at the crest of Greenland the result of climate boundary conditions unique to a glaciated North America, or could they recur tomorrow? Alley *et al.* contribute towards unravelling this problem in two ways. First, they establish the exact age and duration of these climate shifts. Second, they use ice accumulation rate as a simple index of climate change. Knowing the rate and timing of the shifts helps eliminate several postulated mechanisms.

The US Greenland Ice Sheet Project II (GISP2) will complete drilling through the Greenland ice cap sometime this summer. One objective of the GISP2 programme, and the parallel European effort GRIP (Greenland Icecore Project), is to date accurately the apparent shifts from glacial to interglacial conditions that have been observed in previous Greenland ice cores. Alley *et al.* examine the most recent shift to interglacial conditions (Younger Dryas/Preboreal shift) and an earlier aborted shift (Older Dryas/Allerod). Armed with sophisticated sensors to detect subtle chemical and microparticle changes that are known to occur annually, the GISP2 team set out to measure annual layers in the Summit ice core one at a time from the surface to the last glacial period some 18,000 years ago. Compression of the ice and diffusion of the chemical species were expected to obliterate the annual signal beyond this period.

Much to the scientists' surprise, summer conditions left visible annual strata. Bleary-eyed after counting and recounting 15,000 layers, they discovered that the climate shifts occurred in less than a decade and that the shifts occurred approximately 1,000 years earlier than previously estimated from ice cores. Although such layer counting has a cre-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Midnight sunlight shining through fog gives the GISP2 drill tower a halo on summer nights. The frozen hoar frost from the summer fog delimits annual ice bands in the cores.

dibility problem, at the Summit site Alley *et al.* were able to circumvent difficulties that plagued other ice core sites. The ¹⁴C timescale over the deglacial period has been calibrated recently using Th/U/¹⁴C measured in corals and extended ¹⁴C tree-ring chronologies. This calibration gives the basis to compare the ¹⁴C-dated records of the climate shifts to the calendar years as measured by ice-core layering. The match between Alley and colleagues' and independent age estimates of the same shifts seen in lake cores and deep-sea sediments is strong support for their quality.

The ice accumulation rate doubled during the first aborted Older Dryas/Allerod shift and during the final successful Younger Dryas/Preboreal shift from glacial to interglacial conditions. Using modern calibrations, Alley *et al.* estimate that air temperature warmed by 7 °C during these shifts, a value consistent with independent estimates based on the oxygen isotope ratio of the ice. Comparable oxygen isotope shifts have been measured in sediments from Swiss and Polish lakes at precisely the same times. One additional piece of the puzzle falls into place with the new ice-core timescale. The two successive episodes of Northern Hemisphere ice-sheet collapse began at precisely the same time as the climate shifts that are recorded in the Greenland ice core (within the cited uncertainty).

On the face of it, this seems like a happy ending that ties up the loose ends. However, that will require identifying a mechanism for a 7 °C climate warming that reached from North America, across Greenland, the North Atlantic and into central Europe. Analogies to the North Atlantic Oscillation, the much cited ocean-circulation link to climate, cannot apply over such distances. The prevailing theory that these climate shifts were triggered by the switching on of 'thermohaline' density-driven vertical circulation is very appealing; however, its advocates have a difficult, but not impossible, task in explaining mode changes that last only three years. Although areas of deep-water convection are very localized, significant heat release from the ocean to the atmosphere, the control mechanism, requires large areas of ice-free water. How could sea ice come and go so quickly? In fact, an example of such rapid change did occur in the Southern Ocean in the 1970s. More difficult to explain is how the heat release from the thermohaline circulation (down wind of North America) contributed to the North American ice-sheet collapse. We can appeal to nonlinearities in the climate system

and to the concept of climate thresholds, but glaciologists offer an alternative.

The GISP2 ice-core ages align the shifts to warmer climates precisely with the two periods of catastrophic ice-sheet collapse, as documented by the well-dated changes in sea level recorded in Barbados corals. Glaciologists have argued that the two intervals of ice-sheet collapse were more probably driven by some extreme nonlinear response to slowly changing climate than by rapid climate change. Simple diffusion models of the Atlantic Ocean yield surface salinity drops in the North Atlantic of 3–10 per cent during the meltwater pulses associated with the collapse of the ice sheets. This creates a thin surface layer with relatively low thermal heat capacity which allows very rapid summer heating even at subpolar latitudes. With few exceptions, the proxy records of the two climate shifts can be explained by changes in seasonality ascribed to this mechanism.

The strong, rapid shifts described by Alley *et al.* occurred when the climate was sensitized by a long-term shift from glacial to the current interglacial conditions, but whether we should take comfort from that fact and the past 8,000 years of stability is debatable. □

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Yohkoh basks in sunlight

Len Culhane

EIGHTEEN months after the launch of the Japanese Yohkoh ("Sunlight") satellite to study the Sun's corona, astronomers gathered in Sagami-hara to review its principal initial results (*What Have We Learned from Yohkoh Observations?*, 23–25 February 1993). The importance of complicated magnetic field patterns, particularly the 'reconnection' of field lines, for releasing energy in the solar atmosphere was especially evident.

The spacecraft was built by the Institute of Space and Astronautical Science and launched on 30 August 1991. It carries a battery of X-ray instruments provided by Japanese, US and UK research groups¹. Two telescopes image the Sun, one in soft and the other in hard X-rays between 0.2 and 100 keV, with angular resolutions of 2.5 and 5 arcseconds, respectively. High-resolution Bragg spectrometers study selected emission lines to measure the emissivities, temperatures and velocities of plasma from solar flares. Broad-band detectors measure the spectra and variability of flare X- and γ -rays up to 10 MeV.

The Sun produces energy by nuclear fusion reactions in a core of temperature 15 million K. Although energy is transferred outwards initially by radiation, the final transfer to the surface is by convection. Together with differential rotation, this causes a large amount of mechanical energy and magnetic flux to pass through the surface. The resulting magnetic field structures control the state of the corona, a hot, tenuous outer atmosphere where the temperature of the plasma reaches 1–2 million K in large, quiet magnetic structures, 3–5 million K in more compact structures associated with active regions, and up to 50 million K during solar flares. Although the role of magnetic field structures both as channels for energy supply and for containing hot plasma has long been recognized, it seems that Yohkoh may allow a detailed understanding of some processes involved.

An example of magnetic field reconnection supplying energy in large-scale coronal structures was found (D. Sime,

High Altitude Observatory) by comparing Yohkoh Soft X-ray Telescope (SXT) images with visible-light observations in the H α line and from a coronameter at Mauna Loa in Hawaii. Observations from 22 to 26 January 1992 showed a prominence extending above the south-west limb of the Sun on 22 and 23 January. At this time the Yohkoh images showed faint X-ray emission from a



FIG. 1 Superposed images of the solar corona from the Yohkoh Soft X-ray Telescope (red) and the Mauna Loa Observatory coronameter (green). The low-lying helmet streamer, lower right, was formed after a prominence eruption and coronal mass ejection. The yellow (red+green) of the outer part of the streamer shows it to be bright in X-rays.

large closed magnetic field structure above the prominence.

This was also seen by the coronameter, which registers the faint visible light scattered from coronal electrons, using an occulting disk to blank off the strong visible emission from the surface of the Sun. However, between 08:07 and 09:20 UT on 24 January, Yohkoh observations showed that a major change had occurred. Figure 1 shows a large helmet streamer in X-rays (red) and visible light (green/yellow). These images were both obtained at 20:12 UT. The underlying prominence was found to have disappeared, but subsequent

observations on 25 and 26 January showed the newly formed X-ray feature rising at about 1 km s^{-1} .

The disappearance of the prominence indicates that a coronal mass ejection, which blew open the previously closed magnetic field structure, took place before 09:20 UT on 24 January. The re-formation, X-ray brightness and subsequent evolution of the new helmet streamer show that the corona is being heated by reconnection of the disrupted magnetic field. The observations confirm a model² for coronal mass ejections in which magnetic reconnection proceeds outwards along a current sheet that is formed following the prominence eruption. This also shows that the ejections are not necessarily triggered by solar flares, a view that has recently been gaining support³.

On a smaller scale, an example of magnetic reconnection was observed with the SXT following the intersection of two active-region magnetic loops (C. Cheng, US Naval Research Laboratory). In an interval of 20 minutes, a pair of loops intersected, brightened and then re-formed as two parallel structures with the release of about 2×10^{21} joules as heated thermal plasma. A converse example was also seen in which two separate loops brightened and merged.

The SXT has also discovered a new phenomenon associated with active regions, namely the frequent occurrence of transient brightenings (T. Shimizu, Tokyo University). These happen typically every three minutes in regions of high activity; in relatively 'quiet' active regions, the frequency falls to about one an hour. They occur mostly in the form of single or multiple loops, although some are pointlike. The plasma temperature increases from below 5 to around 7 million K; the associated thermal energies are in the range 10^{19} – 10^{22} joules. The brightening of the loops starts either at their footpoints or at sites where several loops are in contact. The footpoint brightenings are suggestive of material being supplied from the chromosphere.



FIG. 2 The development of a twisted jet observed by the SXT between 05:07 and 06:24 UT on 3 October 1991. The jet originated at the site of a compact flare and reached an extension of 220,000 km. The velocity of the top was about 200 km s^{-1} during extension.

Yet another discovery from the SXT is the observation of X-ray jets in the corona. These were first seen in full-frame images with time resolution from minutes to an hour (K. Shibata, National Astronomical Observatory of Japan). They are 5,000–500,000 km long, their tips move at 30–300 km s⁻¹ and their kinetic energies are between 10¹⁸ and 10²¹ joules. They often originate as flares in bright points and emerging flux regions while a void sometimes appears at the footpoint of the jet after ejection. The example in Fig. 2 is of an undulating jet where the material may be expanding along helically twisted magnetic field lines. Shibata *et al.*⁴ propose a number of magnetic field reconnection configurations which can supply energy to drive jets and feed them with material from the chromosphere.

The study of coronal bright points, first observed extensively during the Skylab mission⁵, is also being significantly advanced. The magnetic bipolar nature of the features is now firmly established. In addition a comparison with ground-based magnetograph data shows that magnetic flux decreased in both poles at the time a flare occurred (K. Harvey, Solar Physics Research Corporation). The principal new result to come from this work is that bright-point flares are often associated with increased emission in much larger structures. One example presented involved the production of a jet that moved at 10³ km s⁻¹ and eventually connected with another footpoint 400,000 km away. This site then flared a day later. Here again there is good evidence that reconnection supplies the energy and that interaction of emerging flux with existing coronal magnetic structures is usually involved.

For solar flares there is evidence that magnetic reconnection at the tops of loops is often involved in supplying energy, particularly for larger structures (S. Tsuneta, Tokyo University). The reconnection⁶ is very similar to that described above for the helmet streamer, although time and size scales are smaller. The energy release is followed by an upflow of plasma from the chromosphere. In an event of 16 December 1991, data from the Bragg spectrometer and the Hard X-ray Telescope (HXT) show that plasma flows up following the impulsive deposition of 10²³ joules in the chromosphere by non-thermal electrons (M. Inda, Tokyo University). Alternatively, for the early

phase of the flare of 15 November 1991, data from both telescopes, the Bragg spectrometer and ground-based H α observations indicate that upward flows were driven by thermal conduction (J.-P. Wulser, Hawaii University). However, R. Canfield (Hawaii University) pointed out that both energy transport mechanisms can operate depending on the amount of energy involved⁷.

PHARMACOLOGY

ET touches down in Houston

Timothy D. Warner and John R. Vane

THE three endothelins are highly active peptides — they are the most potent in constricting blood vessels and raising blood pressure^{1,2}, but may also influence such activities as neurotransmitter release, maintenance of electrolyte balance, cell proliferation and hormone production. Snake venom peptides (the sarafotoxins) also belong to the same family³. How goes the research into working out just what the endothelins do and how they do it? Such was the subject for examination at a conference on endothelin, held earlier this year*.

The news was predominantly heartening, as it became plain that investigation of endothelin action is entering a new phase with characterization of the receptors concerned and the advent of selective, potent and orally active receptor antagonists. Conversely, progress in identifying the elusive 'endothelin-converting enzyme' has been frustratingly slow.

Two endothelin receptors are known, the selective ET_A receptor⁴ (endothelin-1=endothelin-2>endothelin-3), and the non-selective ET_B receptor⁵. Both are members of the rhodopsin superfamily with the receptor selectivity being determined by changes in the loop structures, including the location of cysteine residues in loops I and II (B. Haendler, Schering AG; A. Sakamoto, Kyoto University). Although it was thought that ET_A receptors mediate the contractile effects of the endothelins whereas, for instance, vasodilator effects are mediated by ET_B receptors, it is now clear that both receptors can mediate vasoconstriction. For instance, constrictions in the rat aorta, dog coronary artery and saphenous vein, and the iliac artery and saphenous vein of the cynomolgus monkey are mediated by ET_A receptors, whereas the ET_B type mediates constrictions of the rabbit pulmonary artery and saphenous vein, and the dog saphenous vein (D. McMullen and S. Moreland, Bristol-Meyers Squibb). Similarly, it is

Although Yohkoh has not sewn up all of solar physics (no surprise, given the subject's complexity) it is showing where explanations to many of the spectacular coronal phenomena may be found. □

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ET_A receptors that predominantly mediate constrictions of the renal vasculature of the rabbit (P. D'Orleans-Juste, Sherbrooke, Canada), but ET_B receptors are more important in the rat kidney.

So a central issue at the meeting was whether a man is more like a dog, a rat, a pig or some other animal in terms of the distribution of endothelin receptors. Immunohistochemistry provides some clues, but caution must be exercised in drawing conclusions. For instance, in the dog kidney although 80 per cent of the receptors are of the ET_B subtype, vasoconstriction is largely mediated by ET_A receptors (D. Brooks, SmithKline Beecham). This observation also implies that endothelins have other renal effects, such as regulating the responses to vasopressin (D. Kohan, University of Utah).

The roles of the endothelins have been clarified by the development of antagonists such as BQ-123 (ET_A receptor-selective) and PD 145065 (non-selective) (A. Doherty, Parke-Davis). Most notably M. Clozel (Hoffman-LaRoche) reported the development of Ro 46-2005, a non-peptide, orally active, non-receptor-selective antagonist which ameliorates the consequences of severely impaired kidney blood flow and blood clots on the brain. We can also expect increasingly selective antagonists to be developed, for there are effects, such as the stimulation of nitric oxide release from the endothelium, that seem to be mediated by additional receptor subtypes.

So much for the bright side, against which is failure yet to isolate the endothelin-converting enzyme which cleaves the inactive big endothelins (38–41 amino acids) into their active forms (21 amino acids). We do know, for instance, that its minimum substrate is big endothelin (19–34) of which the region 27–34 is most important in determining the velocity of conversion (K. Matsuyama, Banyu, Japan). However, there has still been no definitive characterization of the enzyme. Clearly this could be very important for it is analogous to the development of inhibitors of

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* Third International Conference on Endothelin, Houston, Texas, 15–17 February 1993.

angiotensin-converting enzyme, such as captopril, that stop the production of another potent pressor peptide, angiotension II. This class of drugs has become widely used in treating high blood pressure and congestive heart failure. Interestingly, in the rat uterus big endothelin-1 is only one-seventh as active as endothelin-1 (G. Rae, Florianopolis, Brazil), suggesting that this tissue is a particularly active and/or rich enzyme source, for in isolated vascular preparations there is usually some 100–300-fold difference in potency.

The endothelins are potent releasers of other locally acting agents, such as nitric oxide and prostanoids⁶. It is now evident in increasingly complex systems, such as the dog kidney (D. Miller, Ciba-Geigy) or even in the human hand (D. Webb, University of Edinburgh), that the release of these mediators modulates the actions of endothelin.

There was further evidence for the proliferative effects of the endothelins. For instance, endothelin-1 infusion greatly increases the hyperplasia that follows from balloon-catheter injury of arterial vessels (J. Trachtenberg, Washington University; S. Douglas, Smith-Kline Beecham). So the endothelins may have a major role in atherosclerosis and the reocclusion that often follows surgical opening of occluded arteries.

Of course endothelin should also have its good side, and treatment with endothelin-1 reverses the hypotension associated with endotoxin shock while still maintaining renal function

(A. Lerman, Mayo Clinic).

The next international meeting on endothelins will be held in London in two years' time. What developments can we hope for before then? Top of many people's lists is the characterization of the endothelin-converting enzyme and of the pathway leading to the formation of the endothelins. Although first found as a product of the endothelium, endothelin-immunoreactivity has been detected in a range of other cell types and tissues, which may or may not contain similar processing enzymes. The existence of other endothelin receptors also seems to be increasingly likely; indeed, it may well be difficult to imagine that the actions of three different peptides are mediated by just two receptors. With this in mind it is also important to define better the roles for endothelin-2 and endothelin-3. Do they have physiological or pathological functions? These questions will find answers with the further development of endothelin receptor antagonists for use in chronic, as well as the more usual acute, studies. □

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CHEMICAL DYNAMICS

Chemists make it snappy

Ian W. M. Smith

OVER the past decade, lasers able to generate ultra-short pulses have moved out of the laser physicist's laboratory and onto the chemist's bench. With them, chemists have been able to fulfil a long-standing dream: the direct observation of the motions of atoms as molecules undergo reactions. These advances were celebrated last month in Berlin in what will surely be seen as a landmark conference on femtosecond chemistry*. The 'new frontiers' are opening up not only in molecular science, but also in studies of fast processes in condensed phases and at interfaces.

At first, Heisenberg's uncertainty principle might lead one to suppose that high-resolution information is sacrificed if one works on such short timescales. But this is not so. Experiments typically start with the excitation of an ensemble

of molecules by a femtosecond laser pulse. But because this initial excitation is coherent, high time resolution replaces, in a complementary way, the high frequency resolution of conventional high-resolution spectroscopy.

These aspects of femtosecond spectroscopy are brought out most clearly in respect of molecular vibrations and rotations (A. H. Zewail, California Institute of Technology; P. W. Felker, University of California, Los Angeles). The technique of rotational coherence spectroscopy illustrates particularly well the advantages of working in the time, rather than the frequency, domain — even if some of its most useful applications move us away from the femtosecond world. Large molecules, dimers and clusters have large moments of inertia, and consequently their rotational energy levels are so closely spaced that high-resolution spectroscopy is defeated. But the rota-

tional periods are correspondingly long and for all states are quite simply related to the rotational constants of the molecules. With coherent, polarized light, one can prepare excited species and observe their recurrence times (at which the excited molecules become realigned) and hence measure their rotational periods. From their rotational constants, the structures of the species can be deduced.

In the case of isolated molecules, ions and clusters, much emphasis has been placed on the investigation of unimolecular photodissociation and photoionization. Once a laser has put molecules into a repulsive, electronically excited state, it can take 100–1,000 femtoseconds for the species to dissociate into independent fragments. Clearly, spectroscopic methods based on femtosecond pulses are an appropriate way to observe the unstable dissociating (or transition) states that exist fleetingly as the molecules fall apart.

Transient absorption spectroscopy using femtosecond pulses has been applied very successfully to the photodissociation and predissociation of ICN and NaCl. In Berlin, Y. Chen (University of California, Berkeley) reported the application of stimulated-emission spectroscopy to photodissociation in the very weak visible spectrum of O₃. The experiments employ a white-light probe to stimulate the emission and record a full spectrum at several pump-pulse-probe-pulse delays. This method could probably be applied widely, as it is not limited to strong photodissociating systems and does not, like absorption, require a third state to which excitation is caused in the probe step.

Others recalled the role that emission spectroscopy without this high time resolution can have in uncovering photodissociation. In a simple but elegant form of these latter experiments (J. L. Kinsey, Rice University; D. G. Imrie, Brookhaven Laboratory), conventional, 10–20 nanosecond laser pulses can be used. With experimental care, an extremely weak emission spectrum of the dissociating molecules can be observed, and from this spectrum and how it varies with the excitation frequency, one can deduce the dynamics of the dissociation process. Of course, the quantum yields for such emissions are extremely low, and the experiments require species with inherently strong spectra (less of a limitation with the new stimulated-emission method).

Chemists are interested not only in isolated and dissociating species but also in colliding molecules and their reactions. But even in highly compressed gases, the time between collisions far exceeds the duration of the collisions themselves, making the interaction de-

*Femtosecond Chemistry, Free University of Berlin, 1–4 March 1993.

OBITUARY

Albert B. Sabin (1906–1993)

tails hard to discern. Of course, such difficulties are not present in condensed phases. One can argue that the simplest condensed phase is a van der Waals dimer. The latest application of such species for the investigation of quasi-bimolecular dynamics was described for the reaction between H atoms and CO₂ (C. Wittig, University of Southern California). When HBr, bound in a van der Waals complex with CO₂, is photolysed and the H atom impelled into the CO₂, the delay time for appearance of the OH reaction product varies between 0.25 and 1.5 picoseconds. This delay, and its dependence on the energy imparted to the H atom in the photolysis step, indicate the role played in this reaction by short-lived HOCO species.

New and exciting results were also reported for systems on surfaces, in large clusters and in condensed phases. One such highlight was the study by femtosecond techniques of reorganization at the surface of the semiconductor GaAs (E. Mazur, Harvard University). A sequence of processes could be identified starting with the initial excitation of photons and consequent production of free electrons and changes in the semiconductor's electronic band structure. The resultant destabilization of covalent bonds brings about structural rearrangements on a timescale of 1–10 picoseconds, which under some circumstances can be followed by material leaving the surface.

The meeting was privileged to be given a historical perspective, as well as a view of biological applications of the latest techniques, by George Porter. He charted the progress of time-resolved photochemical methods, from the millisecond flash-photolysis experiments of the 1950s, which he helped pioneer, to the femtosecond pulses of the present day. His lecture posed and prompted the question of whether we have reached the end of a road — can we, need we, go faster?

To follow the motion of atoms in molecules it seems that the time resolution of current techniques may be sufficient, although the development of femtosecond electron diffraction (Zewail) should extend ultrafast measurements to molecules not observable by spectroscopy. The motions of electrons in molecules are characterized by sub-femtosecond timescales. However, light pulses that short will have energy spreads comparable to the strength of chemical bonds, and information that can be acquired is unlikely to throw light on chemical processes. □

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THE heroic era of poliomyelitis research started with the isolation of the polio virus in monkeys by Landsteiner and Popper in 1908, and culminated in the development of vaccines protecting against this paralytic disease in the 1950s. Albert Sabin, who died on 3 March, in Washington, was one of the heroes of this era. His interest in polio is said to have originated during the polio epidemic in New York City in 1931. This was the year Sabin got his MD degree from New York University Medical School.

Sabin's enthusiasm for virology went back to his postdoctoral years at The Rockefeller Institute where he was working under the tutelage of Peter K. Olitsky. Sabin isolated and characterized the herpes B virus which caused the death of scientists bitten by a virus-carrying monkey. With Olitsky, he also studied the factors involved in the neuroinvasiveness of viruses. During the Second World War, as a lieutenant colonel in the US Army, Sabin isolated the virus of sandfly fever and was successful in controlling the infection through the elimination of virus-carrying insects. During the war he was also instrumental in developing vaccines against dengue and Japanese B encephalitis. At one point in his career he became interested in toxoplasmosis and shortly thereafter developed a diagnostic dye test for the disease. These were significant scientific contributions. But, undoubtedly, Sabin's major achievements were in the field of vaccination against polio.

In March 1951, I reported the first immunization of children with live attenuated polio type II virus at a meeting of the National Foundation for Infantile Paralysis in Hershey, Pennsylvania. At that time, Jonas Salk was experimenting with the immunization of monkeys with vaccines made with killed polio virus. Sabin, on the other hand, agreed with me during our discussion at the meeting that a live oral vaccine is a preferable method of immunization against polio. When Sabin developed his attenuated strains of polio he energetically pursued his goal of making them widely accepted as vaccine strains. The most convincing evidence of the innocuousness of the strains and their effectiveness was the vaccination of millions of subjects in 1958 and 1959 in the Soviet Union. This episode, more than anything else, finally convinced the authorities to license a live polio vaccine for immunization in the United States.

Following worldwide use of the oral vaccine, Sabin's time was largely taken up in visiting various countries around the world and acting as an adviser to the polio vaccination programme. At one

point he was involved in research on the possible viral aetiology of human cancer. But he soon became convinced that the herpes virus, which was thought for a while to be associated with cancer, had in reality nothing to do with the disease.

Sabin relentlessly pursued his interest in vaccines. He immunized children against measles by aerosol application, and wrote about the varicella-zoster vaccine. He was also concerned about influenza epidemics and studied the virus involved. He continued writing numerous papers about the oral polio vaccine, with particular emphasis on vaccination problems in Third World tropical countries, and in 1984 he published a description for "Strategies of elimination of poliomyelitis virus in different parts of the world with the use of oral polio virus vaccine". The last case of paralytic polio in the Western Hemisphere was reported earlier this year, from Peru from 1992, a testament to his work.

In the last years of his life, Sabin became interested in AIDS. He was convinced that HIV is transmitted only by mucosal route and doubted whether any vaccine that fails to convey mucosal immunity will protect against the disease. A letter on this topic, a response to some critics, appeared only last month (*Nature* 362, 212; 1993).

That his views on AIDS carried great weight was a result not only of his experience with research on vaccines, but also because he was so articulate and incisive. Sabin was a champion of the debating society at New York University where he graduated. This skill never left him. He was the most eloquent and persuasive participant at meetings and was hard to defeat in scientific argument — not least because he often interpreted data more correctly than the actual presenter.

At one time, Sabin and I became adversaries over the selection of polio virus strains to be used as oral vaccines. This did not affect our long-lasting friendship and mutual respect. In a letter to me written just a year ago, reviewing a paper speculating that AIDS started with polio vaccination in the Belgian Congo, Sabin expressed his opinion that this was "a most irresponsible and uncritical communication". Courageous and wise. This is how I see him. I will miss him sorely.

Hilary Koprowski

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The evolution of virulence

Andrew F. Read and Paul H. Harvey

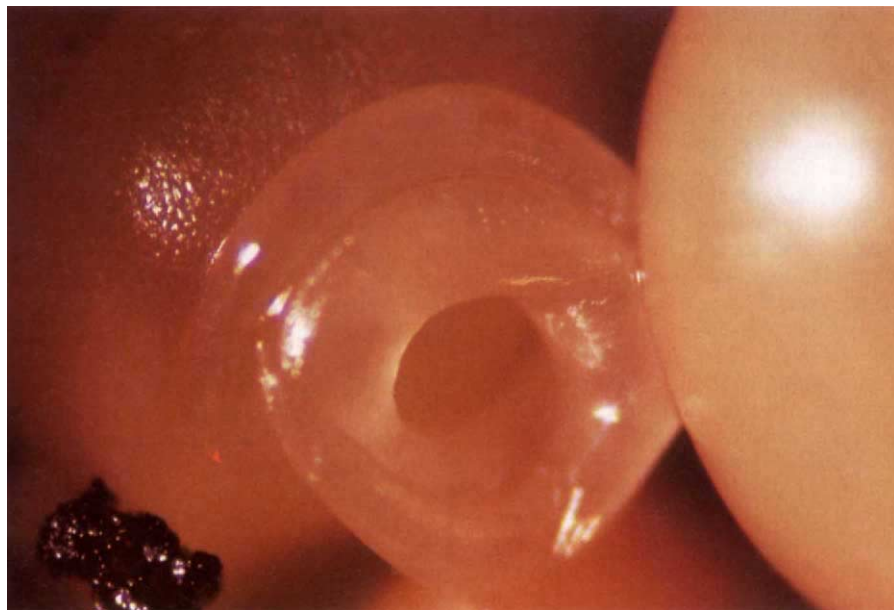
How virulent should we expect animal parasites to be? The answer depends, in part, on whether increased damage to the host improves the reproductive success of the parasite. Biologists have developed a theoretical framework within which the evolution of virulence can be analysed¹⁻⁴, and it has been demonstrated experimentally that virulence can evolve in response to alternative modes of parasite transmission⁵. Now, in an elegant comparative study published in *Science*⁶, Allen Herre shows how differences in the likely mode of transmission correspond with expected differences in virulence in natural populations.

For parasites whose offspring infect the offspring of their host (vertical transmission), any behaviour by the parasite that decreases the fecundity of its host (and thereby that of the parasite itself) is unlikely to be favoured by natural selection. In contrast, parasites whose offspring are not dependent on those of their host (horizontal transmission) are expected to become more virulent if that results in the production of an increased number of parasite young.

In a test of this idea, Bull *et al.*⁵ propagated populations of phage-infected *Escherichia coli* in two ways. In one, phage replication was wholly dependent on host reproduction. In the other, phage could be transmitted vertically and horizontally. The two selection regimes had the expected effects on host fitness: infected bacteria in the vertically transmitted lines increased in density much more quickly than those in the lines where horizontal transmission could occur. Genetic changes in both host and parasite were responsible for the evolved differences in virulence.

The generality of conclusions from well controlled experiments can often be tested by comparative studies⁷. For example, Ewald has argued that variation in virulence among a variety of human diseases may be accounted for by differences in the relationship between virulence and transmission⁸. In his view, directly transmitted diseases such as common colds generally have lower virulence than vector-borne diseases such as malaria because transmission rates in the former are higher if infective hosts continue to come into contact with other individuals. In contrast, host immobility is beneficial to vector-borne diseases because vector-avoidance behaviour is minimized. Ewald's surveys of human diseases⁸ generally fit this pattern. But such broad surveys necessarily include a wide range of parasites, which differ not only in transmission routes but in many

other aspects of their natural history. As one of us wrote only two years ago, "comparisons of the virulence of parasite taxa utilizing different transmission routes from similar hosts are not yet possible"⁹. Herre's study⁶ shows that they now are.



A twist in the tail — *Parasitodiplogaster obtunema*, one of the direct life-cycle nematodes, parasitic on fig wasps, central to the work described here. (Photo, Allen Herre.)

Herre examined a group of direct life-cycle nematodes (*Parasitodiplogaster* spp.) each specific to one of eleven Panamanian fig wasp species (*Pegoscapus* spp., *Tetrapus* sp.). Because of the peculiar natural history of these hosts, the relative importance of horizontal and vertical transmission differs among parasite species.

Gravid female fig wasps enter figs, lay eggs and die. Their progeny mate inside the fig before the young females disperse to new figs. The nematode generations are intimately connected with those of their hosts. For part of their life cycle, nematodes consume the body of the adult female fig wasp. After the death of an infected foundress in a fig, about six or seven nematode adults emerge from the carcass, mate and lay eggs in the same fig. The eggs hatch synchronously with the emergence of the wasp larvae, and the nematode larvae infect young female fig wasps before they disperse. Because the wasps lay all their eggs in a single fig, Herre estimates nematode virulence as the relative lifetime reproductive success of infected compared with uninfected wasps, when each lays on its own in a fig.

A consequence of fig-wasp life-cycles is that the number of female wasps

laying in a fig determines the opportunity for horizontal transmission by the nematodes. If only one female is present, a nematode's offspring must infect the offspring of that host. So selection should favour benign parasites that limit the destruction of their hosts. But where there are more foundresses per fig, parasite reproductive success depends less on the fecundity of its host because other wasps are laying potential hosts. It seems reasonable to assume that greater

consumption of host resources by nematodes causes reductions in host fecundity, and that, as in other parasitic nematodes¹⁰, bigger worms produce more eggs. As a consequence, greater virulence should be favoured if enough offspring of other wasps can be infected to more than compensate for the subsequent loss of extra offspring from the current host.

Herre shows that this is indeed the

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case. In those species where almost all broods are initiated by one foundress, he found that parasitism had no detectable effect on fitness. But in species where less than a third of broods are founded by a single wasp, parasites reduced the reproductive success of their hosts by up to 16 per cent. The relationship between virulence and the frequency of single-foundress broods was roughly linear. Thus, the wasp–nematode relationships ranged from commensal to clearly parasitic, in accord with their different transmission routes.

Comparisons of virulence of closely related parasites in hosts with which they have co-evolved automatically control for many third variables which potentially confound such analyses. Comparisons of the same host–parasite system when transmission is likely to vary, either between populations or over time, may also prove profitable. For example, Dye and Davies¹¹ have suggested that fluctuations in the virulence of gerbil leishmaniasis (an animal model of the human

disease) are a consequence of seasonal variation in transmission dynamics.

Now that observations are beginning to support theories about the relationship between virulence and transmissibility in the evolution of pathogenicity, the way is open for further theoretical developments. Many of the same theoretical issues arise in the study of the evolution of sex ratio in group-structured populations^{1,4,12}, a topic which has spawned some of the most impressive fits between quantitative predictions of evolutionary theory and field data^{13,14}. It is no accident that the curious natural history of fig wasps played an important part in that work as well^{15–17}. □

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heidelbergensis) should be recognized to fill any taxonomic void between *H. erectus* and Neanderthals, and the extent to which internal, rather than between-taxon, variation is responsible for differences between the specimens^{4–7}. On this last point, I have argued that differences between large specimens (such as the Petralona cranium) and small specimens (such as the Steinheim cranium) do warrant a degree of taxonomic separation⁵, whereas others consider that the differences are due more to internal population variation such as sexual dimorphism⁴.

The Atapuerca skeletal sample is large by the standards of any other Middle Pleistocene hominid site, so it provides an unprecedented opportunity to examine the internal morphological and metrical variation of what is assumed to represent a penecontemporaneous sample of individuals of different ages and sex. This is important for questions of taxonomy, because we can observe the relative frequency of *H. erectus* and Neanderthal characters in the sample, and for phylogeny, because questions of character polarity may be resolved (for some parts of the skeleton there were no Middle Pleistocene data before the discovery of the bones at Atapuerca).

The new paper¹ and Table 2 list the extent to which the Atapuerca sample shows some of the characters typical of *H. erectus* (late European), Neanderthals, and modern *H. sapiens*. The absence of most of the distinctive *H. erectus* features in the Atapuerca sample is evi-

PALAEOANTHROPOLOGY

Secrets of the Pit of the Bones

Chris Stringer

SOME 300,000 years ago, the remains of at least 24 people somehow found their way into the Sima de los Huesos, a small chamber deep within a cave in the Sierra de Atapuerca, northern Spain. There they became fossilized. Although the mechanism of accumulation of the jumbled bones presents a puzzle, the discoveries of the latest in a long series of excavations by Spanish workers, reported on page 534 of this issue¹, seem not only to have settled the question of the affinities of the Atapuerca hominids, but also promise to clarify our understanding of the evolution of humans in Europe.

Debate about the European fossil hominid record has concentrated on the Late Pleistocene interface between Neanderthals and early modern humans in the period between about 40,000 and 30,000 years ago (40–30 kyr)^{2,3}. This emphasis has tended to eclipse developments in analysis of the Middle Pleistocene record, which begins with the Mauer (Heidelberg) mandible (estimated age about 500 kyr) and ends at about 130 kyr, after which time undoubted Neanderthals were present in Europe. A possible chronological sequence for some of the more important hominid finds is shown in Table 1, together with proposals as to their taxonomy.

There are two main issues in the study of the Middle Pleistocene sequence. Can

the species *Homo erectus* be recognized in the European fossil record? And how far back can the Neanderthal lineage be traced? Other questions that follow are whether other species (for instance *H.*

TABLE 1. Four interpretations of the European hominid sequence

Age (kyr)	Fossil hominids	a	b	c	d
30	Early modern	<i>H. sapiens sapiens</i>	Modern <i>H. sapiens</i>	<i>H. sapiens</i>	<i>H. sapiens</i>
50	Late Neanderthals	<i>H. sapiens neanderthalensis</i>			
120	Saccopastore				
	Biache			<i>H. neanderthalensis</i>	
220	Ehringsdorf Pontnewydd Reilingen? Swanscombe? Steinheim?	<i>H. sapiens steinheimensis</i>	Archaic <i>H. sapiens</i>		<i>H. neanderthalensis</i>
300	Atapuerca Petralona? Bilzingsleben Vértesszöllös? Arago?	<i>H. erectus</i>		<i>H. heidelbergensis</i>	
500	Mauer?				

Scheme a recognizes two species (*sapiens*, represented by three subspecies, and *erectus*), b only one (*sapiens*, in 'modern' and 'archaic' forms), c three (*sapiens*, *neanderthalensis* and *heidelbergensis*) and d two (*sapiens*, *neanderthalensis*). Assuming that the Mauer mandible belongs taxonomically with the subsequent sample, the new Atapuerca evidence appears to favour scheme b, or d (my own preference).

dent. There are more Neanderthal features present, although some postcranial characters may well turn out to be more widespread plesiomorphies when data from other, non-Neanderthal, samples are known^{8,9}. But the presence of characters such as midfacial projection and an incipient suprainiac fossa does seem

In turn, more fragmentary finds such as those from Vértesszöllös (Hungary) and Bilzingsleben (Germany), regarded by some as representing *H. erectus*, may also be linked morphologically to the Petralona-Atapuerca group. This would lead to the further weakening of claims for the existence of *H. erectus* in Europe

(Table 1, a). The new data also bear upon the concept of a separate species, *H. heidelbergensis*, in the Middle Pleistocene of Europe and Africa^{5,7} (Table 1, c). This concept sprang from the resemblances between European specimens such as Petralona and African crania such as Broken Hill, but it depended not only on a clear demarcation of the specimens from *H. erectus* and modern *H. sapiens* (still very defensible), but also on a demarcation from the Neanderthals. In the case of the European Middle Pleistocene representatives of *H. heidelbergensis*, this second distinction will be much more difficult to maintain following consideration of the variation present in the Atapuerca sample. It is difficult to envisage a suite of characters that could consistently differentiate specimens such as Mauer, Arago and Vértesszöllös from the primitive Neanderthals whose remains are concentrated in the 'Pit of the

Bones' at Atapuerca. The Neanderthal lineage seems to have its roots deep in the Middle Pleistocene. For those such as myself, who believe the Neanderthal lineage was distinct from our own, this would mean that the origin of the *H. sapiens* clade was similarly ancient. □

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TABLE 2. Some character distributions in the Atapuerca sample

	<i>Homo erectus</i>	Neanderthal	<i>Homo sapiens</i>
1. Vault broadest near base	✓		
2. High total prognathism	✓		
3. Laterally thick supr. torus	✓		
4. Mandibular morphology	✓	✓	
5. Lower limb robusticity	✓	✓	
6. Cranial capacity range	✓	✓	✓
7. Shape of supr. torus		✓	
8. Incip. suprainiac fossa		✓	
9. Midfacial projection		✓	
10. Lateral occipital profile		✓	✓
11. Tympanic morphology		✓	✓
12. High cranial vault		✓	✓
13. Shape of temp. squama		✓	✓
14. Rear parietal profile	✓		✓
15. Adult occipmast. morph.			✓

to point to a significant phylogenetic link with the Neanderthals, aligning the Atapuerca hominids with that clade (or species, if *H. neanderthalensis* is recognized).

What of variation in Middle Pleistocene specimens generally? My reasons for distinguishing the Petralona and Steinheim crania included their contrasting facial morphologies and very large size difference. However, the small Atapuerca cranium 5 shows clear facial resemblances to Petralona, while a larger facial fragment (AT-404) displays a resemblance to the cheek region of Steinheim. Size differences between crania 4 and 5 also approach those between Petralona and Steinheim. So although considerable differences remain between the Petralona and Steinheim crania in features such as vault thickness and occipital form, it seems feasible that they can both be regarded as extensions of the variation shown in the early Neanderthal population(s) sampled at Atapuerca.

A bid for pregnancy

IMPORTANT biological objects, as Jim Watson remarked, come in pairs. For example, a woman has two ovaries. Yet every month, only one of them releases an ovum; the other holds back. How do they decide which of them is to do the job that month?

One guess is that they work in strict alternation, perhaps in obedience to a monthly nerve signal from the brain. This seems unlikely; nature is not usually so digital. In any case, reproductive physiology tends to use hormonal rather than nervous signals. Daedalus reckons that the ovaries compete for the monthly honour. Each sends out some sort of hormonal signal, while reading the corresponding signal sent out by the other. Like two poker players, each raises its level of hormonal bidding, until one of them feels outbid, and folds. The victor then provides the ovum for that month.

This mechanism suggests a novel form of contraception. By introducing false signals into the system, it should be possible to trick each ovary into thinking that the other has won the bidding. So DREADCO endocrinologists are taking a regular sequence of blood samples from a panel of female volunteers. They are looking for rapid fluctuations of usually neglected minor steroids during the build-up to ovulation. In these signals they hope to read the bidding and counter bidding of the two ovaries. The results could be quite complex and subtle. Each ovary may well prepare several potential ova each month, like a card player considering which card to play in the light of signals from the opposition. Once the team has cracked this bidding code, they should be able to subvert it. DREADCO's 'Anova' contraceptive will slip a false hormonal bid into the system. It will convince each ovary that the other has won the trick. Both will then fold in defeat, and their owner will not become pregnant that month.

A single monthly pill or injection of Anova should work neatly, with no side-effects. By preventing ovulation it may even suppress menstruation — a useful advantage, once the user has realized that it is not a sign of disastrous contraceptive failure. Furthermore, the same biochemistry might be adapted not to eliminate the chance of pregnancy, but to increase it. For would-be mothers, Daedalus hopes to develop 'Binova'. This inverse hormonal cocktail will be a sort of biochemical misère bid. It will convince each ovary that the other has folded. Both will therefore produce an ovum that month. This will double the subject's chance of pregnancy, and also give her a useful chance of bearing non-identical twins.

David Jones

Yttrium carbide in nanotubes

SIR — Filling the hollow core of carbon nanoclusters with foreign material has added a new aspect to the promise of this family of novel materials. Lead has been inserted into nanotubes by capillary



FIG. 1 High-resolution transmission electron-microscope image of a nanotube with two different sizes of inner cores. Only the larger core is partially filled; the smaller one is empty. Scale bar, 3 nm.

suction through open ends¹, and microcrystals of LaC_2 have been encapsulated into polyhedral cages^{2,3}. We have now used the general approach in refs 2 and 3 to place yttrium carbide into nanotubes.

We prepared samples in a computer-controlled reaction chamber⁴. The composite graphite anode had a centre hole packed with paste of yttrium oxide powder mixed with isopropanol. Both materials were removed in a 550-torr helium arc discharge sustained by 28 V at 70 amps d.c. The material deposited at the cathode was ground into powder for examination by a transmission electron microscope and an X-ray diffractometer.

Figure 1 shows an electron-microscope image of a 10-nm diameter tube contained in the deposit, filled with material in the larger of two internal cavities. The irregular appearance of this material does not necessarily mean that it is amorphous, as lattice images depend on the direction of the electron beam. Apparently, the process conditions promote deposition of the enclosed material in the larger cavity, but prohibit it in the smaller. The inserted material

does not wet the internal cavity surface completely. Filling extends for 100 nm along the length of the tube without overlapping the edges of the innermost tube. This would not happen by chance for material deposited on the outside of the tube. The tube tips remain closed, and show no sign of damage.

Electron energy-loss spectroscopy places the electron beam of nanometer size entirely on the filling material. The M edge of yttrium and the K edge of carbon is observed with no oxygen signal detected (Fig. 2). Yttrium metal peaks are absent in X-ray powder-diffraction patterns. Both results suggest that

the nanotubes are filled with yttrium carbide crystals, and contain no yttrium metal or yttrium oxide.

Low-magnification micrographs show that about 20% of the nanotubes and 50% of the nanoparticles are filled. The

fraction apparently depends on particle shape and dimension, indicating an influence of the internal topography of the cavity. Inclusion in the large diameter cavity but not in the smaller establishes size and shape as factors influencing the ease of filling. Control of the inclusion through variation of the processing parameters will be a critical factor in developing technological applications.

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Chicxulub – K/T melt complexities

SIR — Kring and Boynton¹ attempted to strengthen the link between the Chicxulub structure and the Cretaceous/Tertiary (K/T) boundary by comparing their reconstructed Chicxulub Y6N17 groundmass compositions with typical igneous fractionation trends and impact glasses from the K/T boundary in Haiti. They argue that Y6N17 could not be easily produced by volcanism, and was probably formed by the same impact event as the Haiti glasses.

They reconstructed their Y6N17 compositions from averages of microprobe data and modal analyses of three groundmass minerals, including albite (see figure), asserting that this technique allowed them to avoid the contaminating effects of xenoliths and secondary mineralization that pervade the sample. Regardless of its origin, however, a silicate liquid crystallizing augitic pyroxene in equilibrium with plagioclase of andesine composition would not co-crystallize albite. Typically, albite of this purity results from secondary alteration processes such as hydrothermal metasomatism or authigenic growth during burial diagenesis. In hydrothermally altered igneous rock^{2,3}, pseudomorphic replacement of calcic plagioclase by albite is common, often in cases where relict primary textures are preserved. Corresponding changes in whole rock major-element chemistry (for example SiO_2 , CaO) can cause substantial shifts away from cotectic compositions. Data in Table 1 (of ref. 1) reveal that about 27%

of Kring and Boynton's average Y6N17 groundmass is albite. Consequently, it is possible that alteration is responsible, at least in part, for these unusual groundmass compositions. Whether impact melting is necessary or sufficient to produce these non-cotectic compositions remains to be demonstrated.

Kring and Boynton also state that their Fig. 1b (ref. 1) shows a "coherent mixing trend" defined by slight variations in their reconstructed groundmass compositions (A, B, C and D), and suggest further that the Y6N17 groundmass lies along the same mixing trend as that previously noted⁴ for the Haiti glasses. The notion that the Haiti glass compositions can be modelled as a mixing line⁴ is based on a published range of glass compositions that is much larger than that⁵ used in ref. 1. When additional data⁶ reflecting this more extensive range are considered, a simple collinear relationship between the Y6N17 groundmass and the Haiti glasses is not observed (part a in the figure). In addition, if a mixing relationship exists between the groundmass trend and the Haiti glass trend, it would be evident in any depiction of this projection scheme⁷. However, when these data are projected from olivine (part b in the figure) rather than plagioclase, it is readily apparent that the "coherent (groundmass) mixing trend" (ref. 1) is oblique to the Haiti glass trend.

Is it possible that the groundmass "trend" noted by Kring and Boynton is

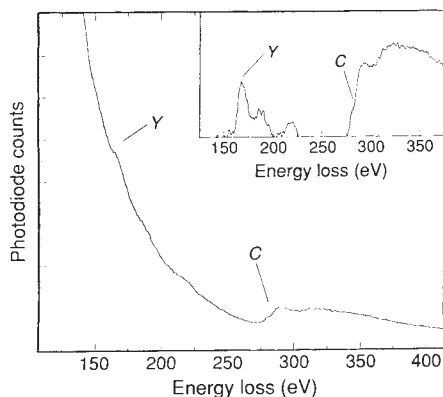
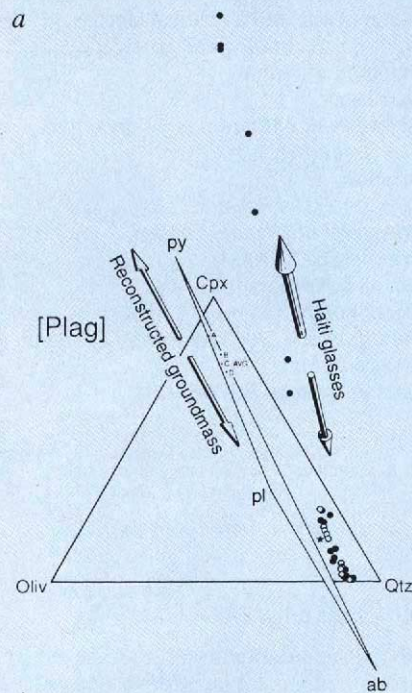
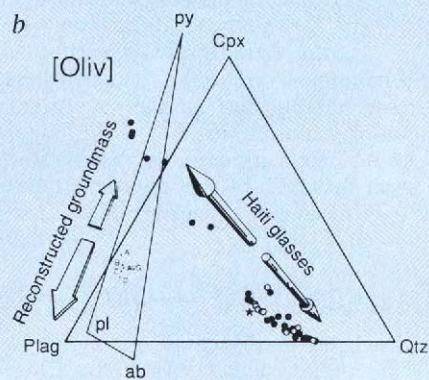


FIG. 2 Electron energy-loss spectra of a nanotube filled with foreign material. Spectrum after removing the background is shown in the inset.

Chemical compositions (mol %) of Chicxulub melt rocks and Haiti tektite-like impact glasses shown on pseudoternary subprojections of the components olivine–clinopyroxene–plagioclase–silica (oliv–cpx–plag–qtz)⁷. a, Projection from plagioclase; b, projection from olivine. Narrow tri-



angular region delimits possible groundmass compositions derived by modal reconstruction based solely on the three minerals considered by Kring and Boynton: pyroxene (py, $\text{Wo}_{44}\text{En}_{43}\text{Fs}_{13}$); plagioclase (pl, $\text{An}_{45}\text{Ab}_{52}\text{Or}_3$); and albite (ab, $\text{An}_1\text{Ab}_{98}\text{Or}_1$). Reconstructed groundmass compositions A, B, C, D and AVG of Y6N17 (ref. 1, unpublished major-element oxide data kindly provided by D. A. Kring). Regression analysis reveals that the modal proportions of pyroxene, plagioclase and albite used to reconstruct the most widely separated compositions (A and D) differ by 6.4, 1.2 and 7.6%, respectively. Haiti glasses (○, ref. 5; ●, ref. 6). Relatively unaltered Chicxulub melt rock C1N10 (★, ref. 8). Schematic arrows approximate the orientation of "trends" for the reconstructed groundmass¹ (flat) and the Haiti glasses^{5,6} (cylindrical).



an artefact? Any composition derived by their technique is constrained to lie within the narrow triangular region of the plane delimited by the three minerals used in their modal reconstruction. It is conceivable that the modest groundmass variations (A–D) derived by Kring and Boynton, on the scale of a thin section, reflect uncertainties inherent in their technique, in which case a "trend" would represent only scatter about an average composition. Furthermore, Y6N17 AVG (see figure) departs significantly not only from the Haiti glass trend, but also from other samples of Chicxulub melt rock⁸, some of which show no petrographic sign of alteration or unmet clasts (see C1N10 in the figure). Consequently, the apparent collinearity of "trends" in their illustration (Fig. 1b in ref. 1), while intriguing, does not provide compelling evidence for a link between Chicxulub and the Haiti glasses.

The fact that this Y6N17 groundmass composition does not lie on the Haiti glass trend does not preclude a genetic association. The Y6N17 melt rock contains abundant partially assimilated clasts of target lithologies¹. Primary variations in whole rock (melt + clasts) composition within a suite of impact melt rocks could reflect variations in the relative proportions of incorporated target lithologies dependent on distance and depth from the impact point. Varia-

tions in the melt matrix or groundmass composition may evolve through selective mechanical disintegration of clasts and non-modal partial melting of minerals to various degrees. Secondary processes that may overprint these primary compositional variations include hydrothermal metamorphism and low temperature alteration over time; large volumes of impact melt may even undergo chemical differentiation analogous to magmatic systems. In contrast to impact melt and melt breccias within and proximal to an impact crater, tektite-like impact glasses are early-formed total melts that are rapidly quenched, and thus more likely to show a less complicated mixing relationship. Given the complexities involved in the formation and evolution of impact melt rocks, there is no reason to expect that tektite-like impact glasses and melt rocks within

the crater will necessarily bear a simple linear mixing relationship.

There is compelling evidence for a link between Chicxulub and the Haiti impact glasses based on $^{40}\text{Ar}/^{39}\text{Ar}$ age determinations^{8–10}. The disparity between groundmass compositions reconstructed by Kring and Boynton and the Haiti glass trend suggests that selective clast melting and alteration are important effects that may not be easily circumvented when attempting to decipher impact melt petrogenesis.

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Properties of an HIV 'vaccine'

SIR — Moore *et al.*¹ raise two points in their Scientific Correspondence. One is that a recombinant gp160 envelope glycoprotein precursor from HIV-1 produced by MicroGeneSys in a baculovirus expression system does not bind strongly to the CD4 receptor; and the other is that this recombinant gp160 does not stimulate the same antibodies as the HIV-1 virus does in natural infection.

We consider these properties to be advantages of our vaccine rather than disadvantages, as it is just these properties that distinguish the MicroGeneSys vaccine from the other candidates. Vaccination with recombinant gp160 in patients infected with HIV-1 broadens HIV-specific envelope-directed immune responses, including crossreactive antibodies to gp160 epitopes^{2,3} and CD4⁺ and CD8⁺ cytotoxic T-cell responses⁴.

HIV is a progressive disease which the immune response ultimately fails to control⁵. Therefore, vaccines that present HIV-1 envelope glycoproteins (gp120 or gp160) in their native form would be unlikely to be of clinical benefit to an HIV-infected individual. By design, we never intended our gp160 molecule to be identical to the native protein.

Antibody responses against native HIV-1 proteins, including the types described by Moore *et al.*, exist in nearly all AIDS patients but do not prevent progression of HIV disease. Also, the binding of gp120 or gp120–antibody complexes to CD4 has been shown to interfere with antigen-specific activation of CD4 cells^{5–7} and trigger programmed cell death *in vitro*^{8,9,11}, which may contribute to the pathogenesis of HIV infection (including immune function deterioration and decline of CD4 T lymphocytes). The absence of CD4 binding by the MicroGeneSys gp160 vac-

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cine may therefore be looked on as an added safety feature.

It is not known which specific immune responses are required for therapeutic benefit, so we have proceeded cautiously. More than 1,100 HIV-positive patients are enrolled in five independent phase I trials and three independent phase II trials with gp160 with up to 4 years of follow-up. The phase I studies have shown evidence of stable CD4 cell counts, stimulation of cytotoxic T cells and the suggestion of restoration of immune function. Some of these results have also been confirmed in phase II studies.

On the basis of these and other clinical results, MicroGeneSys gp160 was chosen by scientists at the Karolinska Institute, the National Bacteriological Laboratory and South Hospital in Sweden for the first phase III vaccine therapy studies.

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SIR — Differences in opinion may exist as to whether the principles of therapeutic vaccines have been solidly established. It is clear, however, that the immune response in preventive and therapeutic vaccines differs in fundamental ways. Preventive vaccines are introduced into an immunologically naive system, whereas therapeutic vaccines encounter a system already immunologically primed. In the present discussion on the gp160 protein from MicroGeneSys¹ this fact has been largely ignored. The concept of 'original antigenic sin' was first shown by studies on the antibody response against influenza virus. A first encounter with a particular virus strain led subsequent contacts with other influenza strains to induce antibody responses strongly biased against the first 'original' strain. Subsequent research has documented that this effect occurs at the B- and T-cell levels.

The fact that gp160 expressed in baculovirus with the sequence of HIV strain IIIB (LAI) will induce different immune responses when used *de novo* in previously unimmunized individuals as compared to already infected individuals is thus highly logical. Whereas non-infected individuals respond with largely type-specific responses, infected individuals respond with a profile distinctly different from the normal individual. A broad antibody response, including not only IIIB but MN and even autologous isolates with regard to neutralization, is thus induced by vaccinating the infected individual. Similarly, there is significant enhancement of T-cell responses against HIV-env-protein-derived peptides.

Our extensive placebo double-blind

study of HIV-1-infected individuals from the Nordic countries and Switzerland that is now starting has two basic aims: (1) To verify or disprove that therapeutic vaccines can induce an anti-HIV immune response of such a kind that it has clinical, positive consequences; and (2) to carry out a double-blind trial that could lead to clinical use of the product.

It has been shown that a broadened serological response occurs after immunization of HIV-infected individuals and that T-cell responses increase drastically². Further, increased affinity to unrelated crossreactive MN HIV peptides increases after immunization with rgp160 of the LAI strain (B. W. *et al.*, unpublished results). These observations suggest that an anamnestic response to the patient's own virus might become reactivated by the foreign, but still related, protein. Certainly other candidates may also induce such reactivities. Both CD4 and CD8 cytotoxic T lymphocytes have, perhaps unexpectedly, been shown specifically to increase during a series of six immunizations of previously HIV-infected subjects⁴.

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MOORE ET AL. REPLY — Volvovitz and Smith claim that MicroGeneSys, Inc. has "designed" its gp160 to be denatured. Oral presentations by company scientists indicate that the design feature involves extraction of gp160 from insect cells in the presence of urea, followed by enrichment of the glycoprotein fraction on lentil lectin columns. The conversion of MicroGeneSys to the virtues of denatured immunogens is one of Damascene proportions. Can this be the same company whose scientists' opinion was only 2 years ago that "... native structure is required for an HIV-1 envelope glycoprotein to be a successful vaccine antigen"¹²? This was at a time when the same gp160 product was being vigorously touted by MicroGeneSys as a prophylactic vaccine candidate; a time when, as now, being able to mimic as closely as possible the natural viral antigens was held to be important for such a vaccine. It is significant that MicroGeneSys gp160 has been excluded from recent trials of prophylactic vaccines¹³. Is it cynical to suppose that, far from "designing" its gp160 to be denatured, MicroGeneSys has rewritten history and grasped the concept of vaccine immunotherapy as a way to exploit their denatured product? *Post hoc ergo propter hoc*.

A rationale for using denatured gp160

as a therapeutic agent in HIV-infected people is to boost the immune response by raising antibodies against novel gp160 epitopes, as Volvovitz and Smith, and Wahren *et al.*, describe above. This concept, originally promoted by Redfield and Birx³, is not entirely devoid of merit. We believe, however, that to achieve a relevant alteration of the immune response will require an immunogen substantially more sophisticated than the MicroGeneSys gp160. It would be a shame if a superficially reasonable idea was destroyed by a premature focus on poorly designed reagents. Wahren *et al.* also justify the use of MicroGeneSys gp160 as a recall antigen, which boosts the production of antibodies raised originally against native viral antigens earlier in infection. But it is precisely those antibodies which Volvovitz and Smith dismiss so cavalierly as unimportant for prevention of progression of HIV disease! Which view prevails?

Volvovitz and Smith cite reports that binding of gp120 to CD4 *in vitro* can perturb immune cell functions. To date, however, there is no evidence from clinical trials that native gp120 vaccines harm the immune systems of naive or HIV-infected recipients *in vivo*. But Volvovitz and Smith fail to point out that the region of retroviral envelope glycoproteins (including HIV-1) most commonly found to have immunosuppressive effects *in vitro* is located in the ectodomain of gp41 (ref. 14), and is therefore present in MicroGeneSys gp160 but absent from gp120. Furthermore, gp160 has the potential for raising unwanted autoimmune responses because of a region similar to MHC class II present in the gp41 moiety¹⁵ but not in gp120. Perhaps these features should also have been "designed" out by MicroGeneSys? The importance under *in vivo* conditions of any of these *in vitro* observations^{5-11,14,15} remains to be determined.

Wahren *et al.* advertise that they are testing MicroGeneSys gp160 in a large-scale clinical trial in Sweden. Data from controlled phase II trials of MicroGeneSys gp160 by the US Army should be available later this year. These will reveal whether or not Wahren *et al.* were wise to expand the scope of their experiment before knowing the results of previous ones. We did not suggest in our previous Scientific Correspondence¹ that such trials should not take place; indeed one of us (J. R.) will soon be involved in a clinical trial of MicroGeneSys gp160 in pregnant women. We restricted our comments to the design of pending trials in the United States that are a matter of considerable debate, and suggested that such trials should be comparative precisely because, as Volvovitz and Smith say, "it is not known which specific

immune responses are required for therapeutic benefit". If only a single product is tested, how are we ever going to know?

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■ See *Nature* **362**, 277; 25 March 1993 for an account of MicroGeneSys's withdrawal of its vaccine from the US Army's trial.

Molluscan shell growth and loss

SIR — Shell growth rings are the principal source of information on the age and growth of molluscs. A growth annulus is assumed to be deposited each year^{1,2}, and microscopic rings are thought to record the length of lunar, daily and tidal cycles¹. The assumption that shell material, once laid down, is never removed, has not been adequately tested. Some studies have used marks on shells that would disappear if shells decrease in size^{3,4}; it is known that molluscs can remain living for long periods without detectable growth⁵; and some studies have recorded negative growth but dismissed it as apparent error. If molluscan shells can decrease in size, then growth rates of molluscs could be overestimated and growth annuli would not reliably estimate age.

We investigated changes in shell size in populations of the common freshwater mussels *Anodonta grandis grandis* and *Lampsilis radiata siliquoides* at two sites

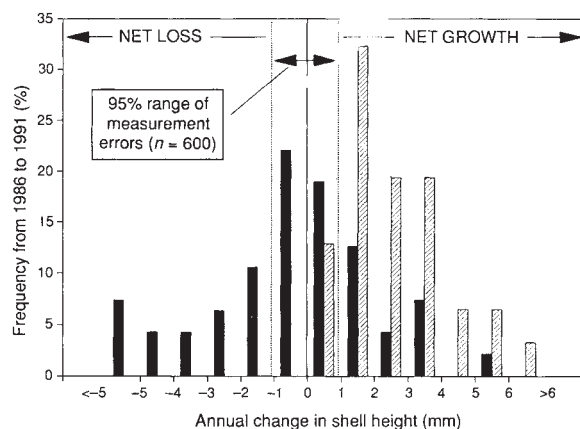
in a well buffered, oligotrophic lake⁶. The rate of shell growth was determined by measuring and marking the edge of the shell at a recorded time, returning the mussels to their natural habitat, then remeasuring the shell dimensions after 1–5 years of *in situ* growth.

Seventy-three *Lampsilis* and 56 *Anodonta* were measured and marked during August 1986 and 1988. We removed animals from the lake briefly, used non-toxic dental cement to glue a pointed, rigid, plastic label at the posterior-ventral margin of one valve of each mussel, and returned animals to the water within 1 hour of collection. Shell dimensions were determined independently by W. L. D. and an experienced field assistant for each mussel relocated during August of 1987–91.

The initial height of each mussel and the height in subsequent years were measured exactly perpendicular to the shell hinge at the umbo using a digital caliper (± 0.01 mm). Mussels were handled gently and kept at lake temperature in ambient lake water during measurement. Measurement error found in blind repeated trials was less than 1 mm in 95% of the 600 trials (see figure).

More than 35% of marked mussels decreased in size. Decreases were commonly 10% and sometimes as much as 20% of total shell height. Although *Lampsilis* showed the most frequent and radical rates of shrinkage, *Anodonta* shells also decreased significantly in size. Decreases in shell size are not due to our marking method or the general water quality in this lake, but vary among sites and populations. For example, *Lampsilis* at the sandbar site grew at rates (average 2.6 mm per yr) similar to those found for the same species in other lakes^{7,8}, whereas less than 1 km away, in the same basin of the same lake, the average rate of growth of marked *Lampsilis* was -0.3 mm per yr.

Changes in shell size were significantly greater than measurement errors (see figure for the example of *Lampsilis*). Most negative measurement errors were between 0 and -1.1 mm; the greatest found in 600 trials was -2.5 mm. At the sandbar site, all *Lampsilis* showed growth, but at the bay site shell height in *Lampsilis* frequently decreased at rates greater than 5 mm per yr. A Kruskal–Wallis comparison of the 52 estimates of annual *Lampsilis* growth that were ≤ 0



Growth rates of *Lampsilis* determined *in situ* by mark and recapture techniques at two sites in Wabana Lake, Minnesota, from 1986 to 1991. Frequencies, number of mussels showing an annual change in shell height that is less than the upper bound of the interval indicated on the abscissa, but greater than or equal to the lower bound of the interval. Vertical broken lines, limits of 95% of all measurement errors determined by remeasuring 10 *Lampsilis* and 10 *Anodonta* of a range of shell sizes 30 times each, in random order. The average estimated size of each shell was calculated and the remeasurement errors estimated as the differences between the average height of each shell and each individual estimate of the height of that shell. Black, bay site ($n=95$); hatched, sandbar site ($n=31$).

with the 247 estimates of measurement error ≤ 0 shows that shell shrinkage is much greater than would be expected from measurement errors alone ($n=299$, $P<0.0001$). Shrinkage was not caused by exterior shell erosion⁹ because the outside periostracum was largely unbroken in shrinking shells and the nacre and crystalline layers inside had been removed, leaving the excess periostracum.

The erasure of shell growth we found has not previously been reported in the longstanding literature on molluscan growth. We hope that people trying to read the shell-growth record will now be aware that part of it may have been erased.

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Cyberscience

William H. Press

Supercomputing and the Transformation of Science. By William J. Kaufmann and Larry L. Smarr. W. H. Freeman (Scientific American Books): 1993. Pp. 238. £18.95, \$32.95.

In their preface, Kaufmann (who has previously written several excellent books) and Smarr (an astrophysicist who now directs the National Center for Supercomputing Applications in Illinois) invoke the image of a global 'cyberspace', a construct of pure information, parallel to, but distinct from, physical reality. Increasingly, we are seeing all sorts of societal activities migrate into cyberspace. The financial sector's move is almost entire. Entertainment and the arts are beginning the transition, not only with digital storage and transmission media (compact disks, high-definition television), but also with the creation of purely computational art, ranging from computer music to the popular 'morphing' of film images.

The intersection of cyberspace with science, variously called computational science, simulation science or (as these authors term it) supercomputing, is a world where physical laws are known or posited, as in theoretical science, but where complex physical systems can be seen (within the computer) evolving in their own only partially controlled manner, very much as in an experimental or observational science. Supercomputing, according to the authors, is not just faster computing. It is marked by the crossing of a distinct threshold in the complexity and realism with which systems of interest are modelled, an abandoning of the simplify-and-idealize tenets of theoretical science in favour of a much richer (though, to some, less pristinely elegant) framework.

A trivial, and at the same time profound, corollary is that the results of supercomputing cannot usefully be displayed as graphs or tables of numbers. Computational science requires, and uses, the most advanced computational techniques of colour graphics, computer visualization and animation. (As part of the Scientific American Library series, this book is lavishly illustrated in full colour. Alas, no accompanying video!)

The first third of the book (three chapters) is an ambitious and generally successful attempt to explain, at a level appropriate to the educated nonscientist, how computational science works, specifically how the continuum (or quantum mechanical) equations that describe physical reality can be accurately approximated by discrete computational techniques such as finite difference, fi-

nite element, Monte Carlo or N-body particle models. The rest of the book surveys the different scientific disciplines and highlights specific projects, mostly work done in the United States at one or another of the national computer centres sponsored by the National Science Foundation. Topics include quark phase transitions, fullerenes, quantum-well devices, chemical kinetics, protein folding, blood flow, brain function, casting and rolling steel, automobile crash testing, mantle convection, ocean circulation, weather and climate prediction, turbulence, astrophysical jets, black holes and large-scale cosmological structure.

Like the rest of the Scientific American Library series, the book is nominally addressed to the adult reader. Notwithstanding, it is an ideal volume for a bright 12–14-year-old, especially one who is (or shows signs of being) addicted to computers. Scientists and science educators might do well to worry about the attractiveness of computers and the sense of power they unlock for brilliant young people today. We in the 'traditional' sciences are perhaps losing too many of the best young minds to in-

terests and careers in computer science and other information technologies.

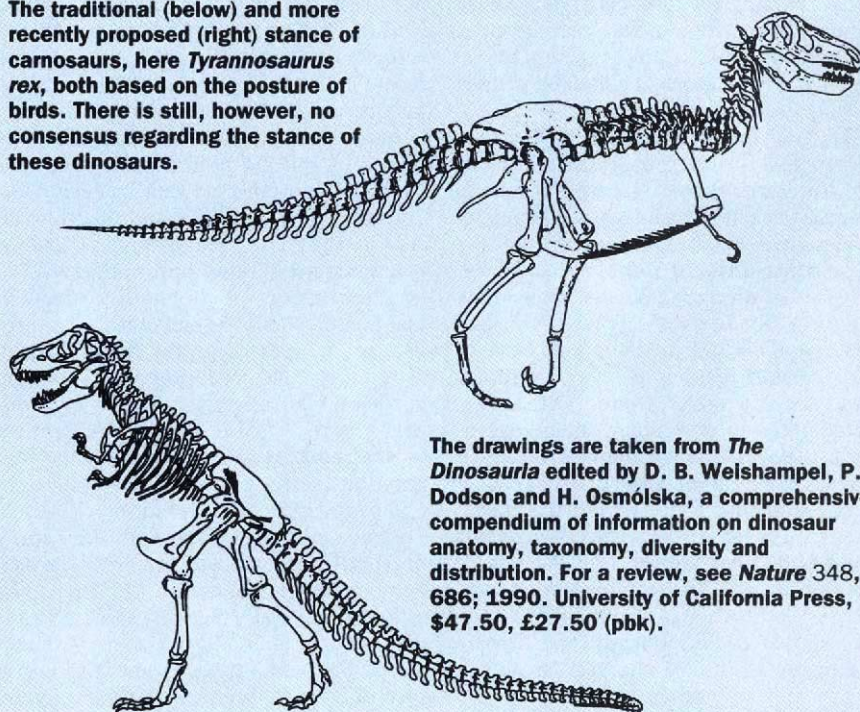
Most of us can name a few particular books, read in pre-college years, that influenced our choice of science as a life's work. The best of these often give a sense of both the science and the doing of science. The younger reader is thus able to get some kind of tentative vision of what his or her own life in science might be. A senior generation of scientists was influenced by Paul De Kruif's *Microbe Hunters* (1926) or Sir James Jeans's *The Universe Around Us* (1929). I recall with pleasure Jane Werner Watson's *The World of Science* (1958), along with the delightful books of George Gamow. In recent years, the books of Paul Davies have inspired many.

Kaufmann and Smarr's book could (one might say, should) be similarly influential to the present generation of youthful computer enthusiasts. Like the books mentioned above, its specific content will be superseded in a few years; but in the meantime the book may be able to rescue some future Nobel laureates from becoming otherwise lost in cyberspace. That is one point of view; another is that these future Nobel laureates may in fact earn their prizes by bringing more supercomputing to science, and thus moving more of science into cyberspace. □

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Good posture

The traditional (below) and more recently proposed (right) stance of carnosaurs, here *Tyrannosaurus rex*, both based on the posture of birds. There is still, however, no consensus regarding the stance of these dinosaurs.



The drawings are taken from *The Dinosauria* edited by D. B. Welshampel, P. Dodson and H. Osmólska, a comprehensive compendium of information on dinosaur anatomy, taxonomy, diversity and distribution. For a review, see *Nature* 348, 686; 1990. University of California Press, \$47.50, £27.50 (pbk).

Fads and friction

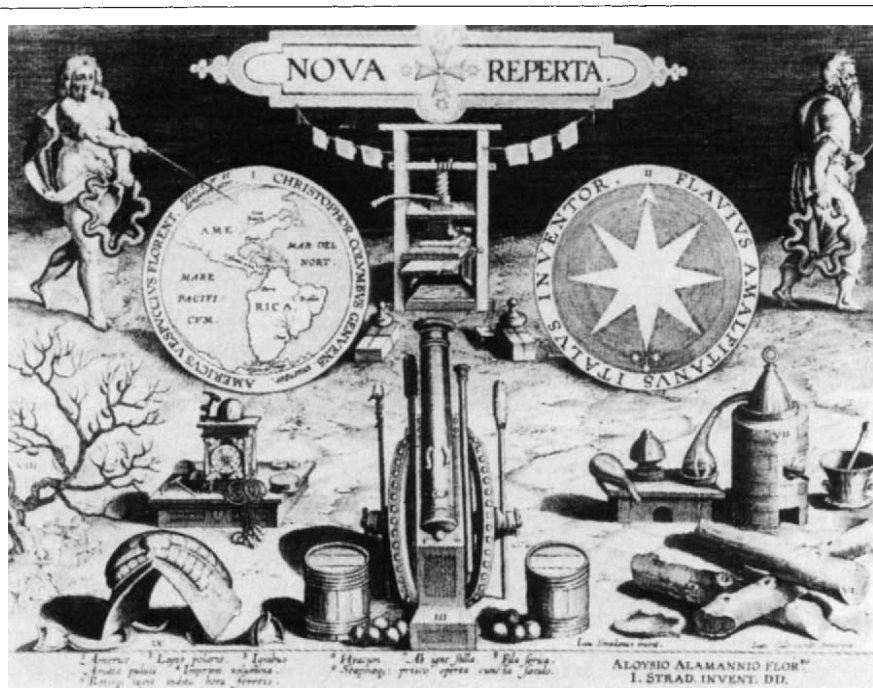
Jacqueline Reynolds

The Culture of Science in France, 1700–1900. By Robert Fox. *Variorum**. 1992. No pagination. £47.50, \$89.95.

SOCIETY in France during the eighteenth and nineteenth centuries was characterized by enormous upheavals, leaps forward and painful retreats. History records the end of the reign of the 'Sun King' Louis XIV, the ineffectual rule of his successor, the brutal singularity of the Revolution, the rise of Napoleon, the restoration of the monarchy, the formation of the Third Republic. And all the while, French scientists continued to do just what they had always done — make new discoveries and expand our understanding of the natural world. This was the period of great excitement at the Jardin du Roi with Buffon, Cuvier and Lamarck, and of that famous duo in Arcueil, Berthollet and Laplace. Lavoisier set the foundation for modern chemistry; Ampère published his monumental treatise on electricity and magnetism; Carnot established the second law of thermodynamics; Fresnel demonstrated the wave nature of light. What a record of achievement!

Robert Fox in his fascination with "the diversity of the contexts in which the French pursued science" has put together 12 of his own papers (all published elsewhere between 1968 and 1985) that describe the changing nature of government influence, scientific institutions and the approach to scientific discovery between 1700 and 1900. There is inevitably a problem with continuity as well as some mildly annoying repetition, but such is the nature of collected studies written so far apart in time.

The first half of the book centres on society and institutions. Fox addresses the attitudes of successive governments towards scientific endeavour, ranging from active encouragement during the Napoleonic days to indifference in the post-Restoration era. We are shown how scientists themselves evolved through these periods both in their approach to research and in their personal attitudes towards fame and fortune. These individual differences, rather than government interventions (or the lack thereof), were in the author's view the real causes of the changing face of French science. The rise of numerous *sociétés savantes* is one of the most interesting aspects of this period, with their accommodation of not just professional scientists but anyone who could be induced to participate and part with the fee charged for annual



ANCIENTS and moderns — an engraving by Johannes Galle of the title page of the Flemish artist Johannes Stradanus's series of *nova reperta*, or new discoveries, drawn in the 1550s. The drawings illustrate silk manufacture and sericulture, the discovery of the New World, lodestone, gunpowder, printing, clocks, distilled alcohol, stirrups, mills and spectacles. This picture appears in *The Mastery of Nature* by T. DaCosta Kaufmann, a collection of essays on art, science and humanism in the Renaissance. Princeton University Press, £30, \$49.50.

membership. Inevitably, these *sociétés* became ever more specialized and less accessible intellectually to the layman, so that by the end of the nineteenth century the universal *savant* had effectively disappeared. Most of Fox's societal framework refers to the provinces and not the better known institutions centralized in Paris, an approach that makes a refreshing change. In fact, one of the most fascinating chapters deals with the history of Mulhouse and the meeting between science and industry in this region of shifting political allegiance far from the centralist powers in Paris.

But all is not politics and institutions. As Goethe said, "The history of science is science itself" and, appropriately, Fox uses the history of the study of heat in eighteenth and nineteenth century France to demonstrate the changing aspirations and preoccupations of the participating scientists. There is Lambert's 'particles of fire' theory of heat set in the context of the arguments that raged at the time: that is, heat as a fluid (caloric) versus heat as motion (kinetic). Dulong and Petit appear with their study of specific heats, the results of which dealt a severe blow to the Laplacian school that supported the caloric theory. The two grand old men of Arcueil, Berthollet and Laplace, and the entire cult of French physics that surrounded them are entertainingly dissected

through the period when French physics moved from uncompromising support of newtonianism to its near rejection with the wave theory of light. (Yes, there were fashions in science even then.) And, finally, Clément, Desormes and Carnot appear on the road to the second law of thermodynamics. Here, Fox endears himself to the working scientist who tires of the seemingly endless efforts by historians to dig up some obscure individual who 'did it first'. In his words, "I believe that, in Carnot's case, it is possible to place too much emphasis on the identification of traditions and precursors. As a result of recent research, we can now trace possible sources for most of the individual elements in his theory, but the originality of the way in which he synthesised these elements remains his unique achievement".

Fox writes for an audience far wider than the historian of science, and although these essays may present some challenge for the uninitiated, today's working scientist (looking beyond the arguments about supercolliders or the effects of retroviruses on the human population) could well profit from a dip into this book. □

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Misinformation

Hermann Haken

Information Theory and Molecular Biology. By Hubert P. Yockey. Cambridge University Press: 1992. Pp. 408. £55, \$69.95.

INFORMATION theory and molecular biology are both large and important fields. In this book, H. P. Yockey tries to link the two. Quite often the word 'information' is used with different meanings, but from the very beginning he sticks to a single interpretation — Shannon information. This information does not carry meaning; rather it is a measure of the scarcity of an event or message. Yockey's starting point is laudable — but how far does it get him?

He begins with a sound presentation of basic principles of probability theory and then discusses the concept of entropy, including that of genetic sequences. There follows a short chapter on the principle of maximum entropy, which regrettably lacks a discussion of the crucial role of constraints. An outline of codes and coding theory provides the main basis for the author's later conclusions. In the chapter on source, transmission and reception of information, he emphasizes the analogies between information transfer and the genetic code.

It is in the second part of the book that Yockey extends this theory to problems in molecular biology. He begins by presenting the information content of several protein families and goes on to deal with a variety of subjects, such as the evolution of the genetic code, neurobiology, the primaeval soup, black holes and cosmology.

In several places the author states that although molecular biology must be consistent with chemical and physical processes, biological principles cannot be derived from physics and chemistry alone. This statement holds still more for the relationship between mathematics and molecular biology (or any other branch of science). But Yockey repeatedly conveys the impression that the laws of molecular biology can be derived from mathematics. In addition, information theory by itself does not contain any dynamics: in order to understand the genetic code, the study of the underlying dynamics of its self-organization is essential. So the author's polemic against the seminal work of Manfred Eigen, who clearly recognized and formulated the fundamental role of these dynamics, is not only unfounded but misleading.

Undoubtedly, information theory has its uses in molecular biology, in particular by giving insights into the way genes

code for proteins. But instead of sticking to this problem, which is interesting in itself, Yockey makes wild extrapolations in a futile attempt to show how the classical concept of information can be applied to problems in *generating* information. He would have been wiser to have published only the first part of this book. □

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Elusive concept

Michael E. Akam

Evolutionary Developmental Biology. By Brian K. Hall. Chapman and Hall: 1992. Pp. 275. £29.95, £47.50.

WHEN walking across the upland fells of Britain, it is not uncommon to find the whitened jawbone of a long-dead sheep, teeth rattling in its sockets. Food for thought on a wet day, for both the developmental and evolutionary biologist. What mechanisms built the jaw's distinctive pattern, each tooth unique, the bone an asymmetric array of lumps and bumps? What mix of selective forces and developmental constraints moulded the mandible, honed the teeth? Here, *par excellence*, is the meeting of the ways for developmental and evolutionary biology.

The mandible is a favourite subject for Hall. His discussion of the forces that shape it is one of the best parts of his book. He approaches his theme — the interplay of developmental and evolutionary biology — first as historian, then reviewer and finally, in the context of the mammalian mandible, as modeller.

As an annotated bibliography, the book is superb. There are nearly a thousand references, spanning the literature from palaeontology to molecular biology, much of it recent, but with good coverage of the eighteenth and nineteenth centuries (12 references for Geoffroy Saint-Hilaire!) and, perhaps most valuable, a survey of the extensive but scattered literature of the twentieth century. Here you will find pointers to classic studies, still pertinent, but not accessible from compact disc databases or minireviews: Waddington on developmental canalization, Gilbert on cyclo-morphosis, and Etheridge on kidney development.

But the book aims to be more. It aims to re-examine key concepts — hierarchy, epigenesis, archetype, homology. As a developmental geneticist, I hoped for a personal synthesis of the subject viewed

from a different perspective. For the first 160 pages I was disappointed. Haeckel's views, de Beer's definitions, Waddington's ideas, yes; but only glimpses of a personal perspective. These chapters are not easy to read. Sentences running to 70 or so words don't help. The ideas shine out most clearly from the frequent and well chosen quotations: Owen — "Homologue The same organ in different animals under every variety of form and function. Analogue A part or organ in one animal that has the same function as another part or organ in a different animal" (1843); Bateson (on late nineteenth century biology) — "Morphology was studied because it was the material believed to be the most favourable for the elucidation of the problems of evolution, and we all thought that in embryology the quintessence of morphological truth was most palpably presented" (1922); Whitman (on developmental constraint) — "But if organisation and the laws of development exclude some lines of variation and favour others, there is certainly nothing supernatural in this, and nothing which is incompatible with natural selection" (1919). Pithy stuff. But elsewhere, if the original author is not quoted, the ideas often fail to jump off the page.

It is too easy to criticize a book of this scope for errors of detail. But in places, Hall fails to do justice to the very logic of the subject. Under the heading "Key innovations as single gene mutations" we find a discussion of torsion in gastropods. Sturtevant showed in 1923 that the handedness of coiling in snails is controlled by a single pair of genes. Hall seems to endorse a proposal made some years ago by Stanley that, on the basis of this simple genetic control, "Torsion, and with it the class Gastropoda, arose through a single gene mutation." It is a profound error to equate a mutation that changes the symmetry of torsion with a mutation that invents the mechanism of torsion itself. This is like saying that all the genetic differences between male and female, oogenesis and spermatogenesis, are specified by a single gene on the Y chromosome. This may be true at the population genetic level, but it tells us absolutely nothing about the developmental complexity of the process itself. This error is common enough in the literature, but it is disturbing here to find even a hint of support for such thinking.

The book becomes gripping once Hall allows his own interests and opinions to come closer to the surface. Does the idea of homology apply only to morphological patterns, he asks, or can it be applied to developmental processes? He follows the topic from Darwin and E. B. Wilson to Hinchcliffe, Oster, Roth, Alberch and Wagner. For once, we are

left in no doubt where the author's sympathies lie, and why — "Homology is a statement about pattern, and should not be conflated with a concept about processes and mechanisms". Before I read the book, I thought this was exactly the opposite of the truth. Now I am not so sure. I appreciate more clearly that, to quote another of Hall's quotes, "Among evolutionary biologists, homology has a firm reputation as an elusive concept" (Wagner, 1989).

For anyone with an interest in the subject, Hall's book is a valuable guide to who thought what, and where to find it. But don't use it as a primer. □

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Time warp

Oliver Penrose

Time's Arrow: The Origins of Thermodynamic Behaviour. By Michael C. Mackey. Springer: 1992. Pp. 175. DM98, £40, \$49.

THE second law of thermodynamics always arouses interest because it is the only widely applicable law of physics that is not symmetric under time reversal, and so singles out a particular direction of time. The law states that if a physical system is thermally isolated (that is, if no heat enters or leaves the system), the thermodynamic quantity known as entropy must increase or stay constant. The law therefore singles out the time direction of irreversible physical processes as the direction of increasing entropy.

For example, imagine two cylinders of gas joined by a narrow pipe containing a valve that is at first closed, then open for a short time, then closed again. Somebody measures how the gas is distributed between the cylinders before the valve opens and after it closes, writes the results on two cards, and asks you to tell which card describes the earlier state and which the later. One way of solving the puzzle would be to use the second law. It is not difficult to show that (other things being equal) the more evenly the gas is distributed between the two containers, the greater the entropy; so the state with the gas more evenly distributed between the two cylinders is the one that occurred later.

When the second law was originally put forward by the founders of thermodynamics, it must have seemed completely mysterious. Unlike the other concepts of thermodynamics such as energy,

temperature, pressure and so on, entropy had no counterpart in mechanics or everyday experience. But in 1872 Ludwig Boltzmann discovered one of the jewels of nineteenth-century physics, a precise quantitative relation giving the entropy of a gas in terms of mechanical quantities, namely the distribution of positions and velocities of its molecules. He showed, in his famous 'H theorem', that if the gas is not in equilibrium then collisions will increase the entropy until finally a state of equilibrium is reached.

Around the turn of the century, J. Willard Gibbs discovered another important formula connecting entropy with mechanical quantities. Unlike Boltzmann's formula for the entropy of a gas, that of Gibbs applies to liquids and solids as well; but it pays for this greater generality by being applicable unambiguously only to systems that are in equilibrium.

In real life — or even in the physics laboratory — we frequently encounter objects that are neither gases nor in equilibrium. One would like to have a 'time's arrow' covering this more general case too — that is, a formula for the entropy of a non-gaseous non-equilibrium system that can be shown to have the property of non-decrease in time if the system is thermally isolated. Neither of the formulas so far mentioned will do: the Gibbs formula gives an entropy that for an isolated system of molecules is easily shown to stay constant in time rather than to increase, and the Boltzmann formula also does not have the desired non-decrease property if the system is not a gas.

The title of this book leads one to expect a contribution to the solution of this problem, but unfortunately these hopes are soon dashed. The discussion is based on what the author calls the "Boltzmann-Gibbs" entropy, but in the terminology used above it is just the Gibbs entropy. His claim in Chapter 1 to prove that this is essentially the only possible definition is belied by the existence of Boltzmann's definition, which is certainly different. The author notes that the entropy, as he has defined it, is constant for systems whose time evolution is governed by differential equations such as the laws of newtonian mechanics. Because he wants the entropy to increase with time, the obvious conclusion would be that the wrong definition of entropy has been chosen and that it should be changed, but he decides instead to change the law of time evolution. The change he makes is to assume that the laws of motion obeyed by the particles constituting an isolated system are non-invertible: that is, that the same dynamical state at a given time can be reached from two or more different dynamical states at some earlier time.

Because the generally accepted laws of motion for such a system of particles do not have this property, his assumption is a departure from what we know about physical reality.

The rest of the book is an exploration of the consequences of this unphysical assumption. The hypothetical world where this assumption holds contains, indeed, many interesting things, and the author guides us around it skilfully. Unlike some mathematical authors, he does not write as though his prime objective is to show how clever he is; he provides plenty of examples to illustrate his theorems and definitions, and clear summaries at the end of each chapter. All the book needs to make it worthy of recommendation as an introduction to this enjoyable branch of mathematics is a health warning like the one on the tobacco advertisements: "Reading this book can damage your understanding of physical reality". □

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New in paperback

■ **Understanding the Present: Science and the Soul of Modern Man** by Bryan Appleyard. This book was the cause of considerable controversy when first published in the United Kingdom last year. The author's central argument is that "Science is not a neutral or innocent commodity . . . a convenience. Rather, it is spiritually corrosive, burning away ancient authorities and traditions". *Nature's* commentators were not convinced — for reviews see *Nature* **356**, 729 and **357**, 29; 1992. Picador, £6.99. (US hardback edition recently published by Doubleday, \$23.50.)

■ **Vital Circuits: On Pumps, Pipes and the Workings of Circulatory Systems** by Steven Vogel. In a review in *Nature* **358**, 720 (1992), David Weatherall wrote that "[Vogel] leads us through the functions and regulation of the heart and circulation with extraordinary clarity and lightness of touch. It is difficult to find fault with this eccentric and lively account". Oxford University Press, \$12.95.

Spring Books

Next week's issue (15 April) contains *Nature's* Spring Books supplement. Among featured reviewers will be Steve Blinkhorn on the psychology of interrogations, confessions and testimony; Fred Hoyle on the works of Copernicus and Kepler; L. Sprague de Camp on polar mythology; John Mollon on Vincent van Gogh, chemicals and creativity; and Alison Jolly on chimpanzees and humans.

Largest subunit of *Drosophila* transcription factor IID directs assembly of a complex containing TBP and a coactivator

Robert O. J. Weinzierl, Brian David Dynlacht* & Robert Tjian

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The TFIID complex consists of the TATA-binding protein (TBP) and associated factors (TAFs) serving to mediate transcriptional activation by promoter-specific regulators. Here we report the cloning of *Drosophila* TAF_{II}250 and the assembly of a partial complex containing recombinant TBP, TAF_{II}110 and the C-terminal domain of TAF_{II}250. This triple complex supports Sp1 activation and reveals specific interactions between TAF_{II}250, TBP and TAF_{II}110.

REGULATED transcription requires specific interactions between activators bound to promoter sequences and other components of the RNA polymerase transcriptional machinery (reviewed in refs 1, 2). Previous studies have suggested that one particular RNA pol II transcription factor, TFIID, is a likely target for interactions with activation domains of promoter-specific transcription factors³⁻⁷. *Drosophila* TFIID is a multiprotein complex consisting of the TATA-binding protein (TBP) and at least seven TBP-associated factors (TAFs) ranging in size from 30K to 250K^{8,9}. Although TBP alone can sustain a basal rate of transcription, TAFs are necessary to direct activator-dependent transcription⁸⁻¹². Thus, some of the TAFs are expected to function as coactivators, a new class of transcription factors that may mediate activation and/or repression by interacting directly with sequence-specific transcriptional regulators^{1,6,8,10,11}. Other TAFs may serve as bridging proteins that allow different coactivators to assemble into a complex containing TBP, a subunit common to RNA pol I, II and III initiation complexes¹³⁻¹⁸. Additional TAFs in the TBP/TAF complex may interact with some components of the general transcription apparatus, possibly RNA polymerase itself or other basal factors.

The structure, function and interactions between different TAFs, therefore, are of substantial interest in sorting out the molecular mechanisms governing these novel transcription factors. We have begun to purify various TAF proteins and clone the genes encoding each of the subunits in order to obtain biochemically useful quantities of purified material. These reagents will in turn help us to gain insights into the function and mode of action of TAFs, and their relationship to each other within the TFIID complex. To identify one or more TAFs that might function as coactivators, we recently cloned the gene for *Drosophila* TAF_{II}110 and found that this protein interacts specifically with the activation domains of Sp1 (ref. 9). Although dTAF_{II}110 has many of the properties expected of a coactivator, we were unable to demonstrate any direct interaction between it and dTBP. Indeed, until recently we were unsuccessful in our attempts to reassemble any of the cloned and expressed TAFs into multiprotein complexes containing TBP. Apparently a key component of TFIID that links various TAFs and putative coactivators such as dTAF_{II}110 to the basal transcription factor TBP was missing.

Here we describe the molecular cloning and initial functional analysis of the largest subunit of the *Drosophila* TFIID complex, dTAF_{II}250. We have expressed a C-terminal, truncated version

of dTAF_{II}250 (Δ N250) and tested the ability of this truncated product to interact with the TATA-binding protein *in vitro*. In addition, we have assembled a partial complex containing recombinant Δ N250, dTBP, and dTAF_{II}110. These recombinant proteins were subsequently tested for potential coactivator function by *in vitro* transcription. Our studies suggest that dTAF_{II}250 provides a direct link between coactivators such as dTAF_{II}110 and the basal transcription factor dTBP and thus might play a central role in coordinating and regulating the assembly of the TFIID complex *in vivo*.

Detection of direct TBP-TAF interactions

As previous studies have shown that hTAF_{II}250 interacts directly with hTBP^{19,20}, we probed a blot containing renatured TAFs using a ³²P-labelled fusion protein of dTBP with glutathione *S*-transferase (GST). Although all seven TAFs were present on the blot, we only obtained one strong signal that coincided with the position of dTAF_{II}250 and a breakdown or alternatively processed product of this high-molecular-weight protein (Fig. 1c). Further experiments revealed that a truncated version of dTBP, consisting of the highly conserved C-terminal domain, is sufficient to mediate this interaction (Fig. 1c). We also tested other fractions containing basal factors^{8,21}, including TFIIB/E/F and RNA polymerase II, and failed to detect any proteins that bind directly to TBP by this stringent assay (Fig. 1c). These findings indicate that TBP and TAF_{II}250 interact directly and that TAF_{II}250 is present in the TFIID fraction but absent in fractions containing TFIIB, E, F or RNA polymerase II.

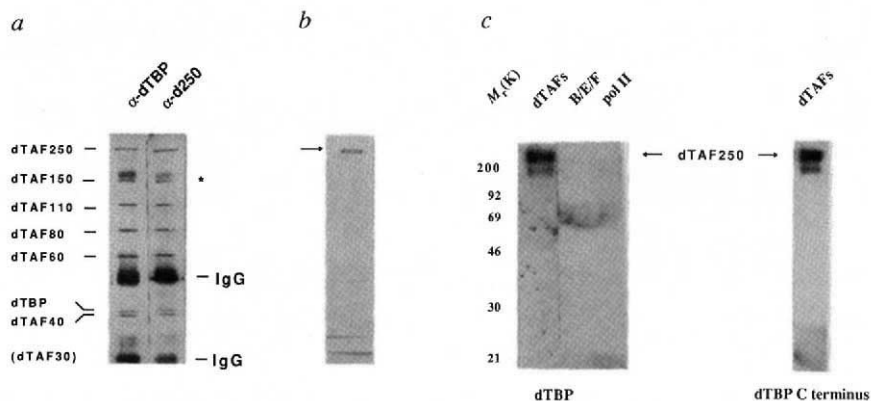
Cloning and characterization of dTAF_{II}250

The low abundance and large size of dTAF_{II}250 does not favour cloning strategies based on protein purification and microsequencing. Instead, we have relied on using specific antibodies directed against dTAF_{II}250 (Fig. 1b) to isolate the gene. To confirm that dTAF_{II}250 is indeed a genuine component of the TFIID complex, we used two monoclonal antibodies, 2B2 and 30H9, to carry out immunoprecipitations from the TFIID fraction (Fig. 1a). The pattern and stoichiometry of TAFs and TBP is indistinguishable from immunopurified TFIID complexes obtained previously using either anti-dTBP⁸ or anti-dTAF_{II}110 (ref. 9) antibodies. A λ gt11 expression library prepared from 9-12-hour-old embryos was screened²² with hybridoma supernatants containing either 2B2 or 30H9 anti-dTAF_{II}250 monoclonal antibodies. Five partial complementary DNA clones were obtained, which all cross-hybridized with each other at high stringency. Restriction mapping and sequence analysis confirmed that they were indeed derived from the same gene. Two of these cDNAs, λ D-1 and λ D-2, allowed us to establish

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FIG. 1 dTAF_{II}250, an integral component of TFIID, interacts directly with dTBP. **a**, Monoclonal antibodies directed against dTBP (antibody 42A11; ref. 9) or dTAF_{II}250 (antibody 2B2) were used to immunoprecipitate the TBP-TAF complex from the TFIID (Q.3) fraction⁸. The patterns are identical, with both antibodies, indicating that dTAF_{II}250 is present in most or all TBP/TAF complexes. The molecular weights of individual dTAFs are indicated. A fuzzy band migrating just above dTAF_{II}150 (indicated by an asterisk) is a contaminating protein derived from the hybridoma cell culture medium and therefore does not correspond to a TAF. dTAF_{II}30 co-migrates on this gel with the light chain of the antibodies. **b**, A crude nuclear extract was probed with anti-dTAF_{II}250 monoclonal antibody 2B2, one of the antibodies used to isolate dTAF_{II}250 cDNA clones from a λ gt11 library by expression screening. 2B2 is highly specific for dTAF_{II}250 (arrow). **c**, A western blot containing immunopurified TFIID complex components (as shown in **a**) and other nuclear fractions (TFII B/E/F and RNA polymerase II) was probed with ³²P-labelled dTBP-GST fusion protein²⁸. The signal, present exclusively in purified TFIID, corresponds to the position of the largest TAF, dTAF_{II}250. A similar blot (shown on the right panel) was probed with a GST-fusion protein containing the C-terminal evolutionarily conserved domain of *Drosophila* TBP (191C; ref. 29). The signal is indistinguishable from the one obtained with full-length dTBP proving that the C-terminal domain is sufficient to mediate the interaction between dTBP and TAF_{II}250.

METHODS. Protein blot (far-western) analysis: to generate an expression



clone for producing dTBP-GST fusion protein, pGEX-2TK (ref. 28) was linearized with *Sma*I, phosphatase-treated and then ligated with gel-purified *Nde*I fragments of either pARdTFIID or pARdTFIID-191C (ref. 29). The generation of ³²P-labelled GST fusion protein, protein blotting and hybridization were all essentially as described²⁸. Anti-dTAF_{II}250 hybridoma cell lines were derived as in ref. 9. Briefly, a Swiss-Webster mouse was immunized with intact immunopurified *Drosophila* TFIID complex. After fusion hybridoma supernatants containing anti-dTAF_{II}250 antibodies were selected using strip-blot containing SDS gel-separated TBP and TAFs. Two such cell lines, 2B2 and 30H9, were then cloned to homogeneity by limited dilution. Immunoprecipitations were done as described⁸, except that protein G beads (Pharmacia) were used to immobilize the antibodies.

a composite open reading frame spanning 4.5 kilobases (kb) (Fig. 2). Attempts to isolate additional cDNA clones encoding N-terminal regions of dTAF_{II}250 have so far been unsuccessful. Genomic DNA sequencing allowed us to extend the open reading frame towards the N-terminus by ~0.5 kb before encountering non-coding (presumably intronic) sequences.

Inspection of the open reading frame encoded by the cDNA clones (Fig. 3a) reveals that it is 60% homologous over 1,000 amino acids to hTAF_{II}250/CCG1 (refs 20, 23). An alignment of dTAF_{II}250 amino-acid sequences with hTAF_{II}250/CCG1 reveals a significant number of conserved structural motifs (Figs 2 and 3a), such as the direct repeats of 120 amino acids and a highly acidic domain (Fig. 3a). The putative HMG box in hTAF_{II}250/CCG1 (refs 20, 23) may not be functional, because the *Drosophila* protein contains a 34-amino-acid insertion within this domain which would disrupt the conserved region (Fig. 3b). It is possible that there are multiple spliced or processed versions of the TAF_{II}250 (ref. 20), and therefore the cDNAs we have isolated may not represent the only or major species.

We have also used the λ D-2 cDNA fragment to map the dTAF_{II}250 gene to position 84A2-3 (right arm of chromosome III) by *in situ* hybridization. This locus does not contain any previously characterized *Drosophila* genes and at present there are no deletions known that span this region. Because dTAF-250 seems to be present in all or the majority of the TFIID complexes present within cells and seems to provide essential contact points with TBP, probably a deletion of the 84A2-3 locus would cause a lethal phenotype.

Portion of dTAF_{II}250 sufficient for TBP binding

To study the functional properties of dTAF_{II}250, we produced the protein encoded by one of our longest cDNAs, λ D-1, in a baculovirus expression system. We detected a 180K protein (subsequently referred to as Δ N250) that crossreacted strongly with several anti-dTAF_{II}250 monoclonal antibodies recognizing a variety of epitopes of dTAF_{II}250 (Fig. 4a; R.O.J.W., unpublished results). We detected no crossreactivity between our antibodies and any endogenous TAF_{II}250 homologues that might be present in the Sf9 cells used for producing the recombinant protein. As we wished to address the nature of the interaction

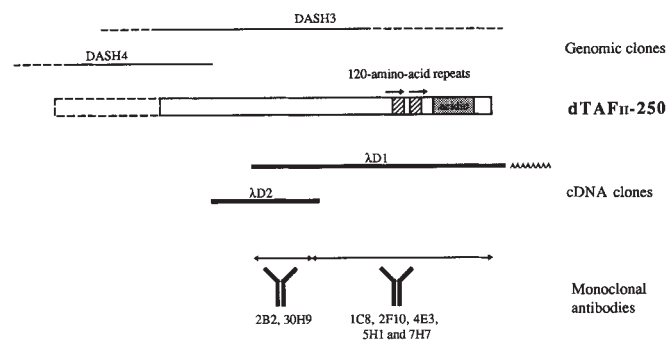


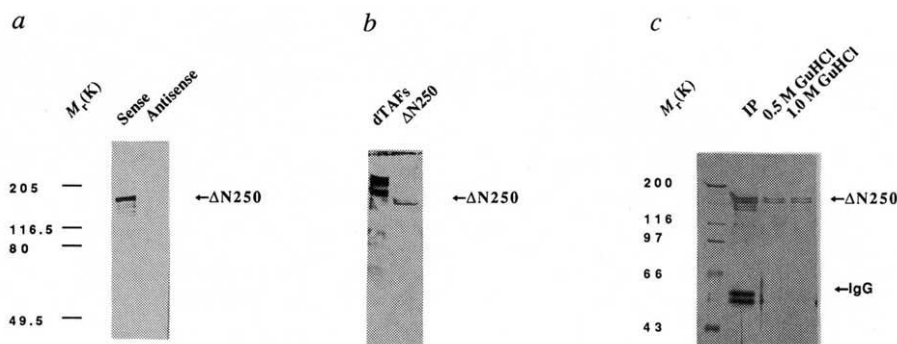
FIG. 2 Schematic diagram showing the position of dTAF_{II}250 cDNAs, genomic clones and epitopes of monoclonal antibodies. Two overlapping cDNA clones, λ D1 and λ D2, define a composite open reading frame for the C-terminal portion of dTAF-250. The sequence was extended towards the N terminus by sequencing genomic fragments derived from clones λ DASH3 and λ DASH4. The two monoclonal antibodies (2B2 and 30H9) used to isolate cDNA clones from a λ gt11 library³⁰ recognize the protein portion encoded by the λ D2, whereas other monoclonals have epitopes located in the C-terminal part of the protein encoded by λ D1 (R.O.J.W., data not shown). **METHODS.** About 5×10^5 independent plaques of a size-selected (≥ 1.8 kb) *Drosophila* λ gt11 library prepared from *Drosophila* embryos²² were screened with two independent anti-dTAF_{II}250K monoclonal antibodies, 2B2 and 30H9. All the positives identified cross-hybridized at high stringency with each other on the DNA level. Restriction mapping and sequence analysis showed that all of the clones were derived from the same gene. cDNA clones λ D1 and λ D2 contained inserts of 1.5 and 4.0 kb, respectively, and were sequenced to completion. λ D2 was found to extend 500 bp further towards the 5' end of the gene and was used to isolate genomic clones λ DASH3 and λ DASH4 (*Sau*3A partially digested DNA cloned into λ DASH; A. Kolodkin, unpublished results).

of TAFs with TBP, we next tested whether Δ N250 could interact with dTBP. Like full-length dTAF_{II}250, Δ N250 retains the ability to bind to dTBP specifically (Fig. 4b), as determined by far-western blot analysis. The capacity of Δ N250 to bind dTBP indicates that the cloned C-terminal 180K contains interfaces necessary and sufficient to interact with dTBP.

b

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FIG. 4 Production and purification of a recombinant version of dTAF_{II}250, Δ N250. **a**, The cDNA insert of λ D1 was clones in both orientation into a baculovirus expression system and introduced into Sf9 cells. Recombinant virus containing the DNA in the 'sense' orientation causes the production of the C-terminal portion of dTAF_{II}250, Δ N250, which migrates with an apparent M_r of 180K and is specifically recognized by anti-dTAF_{II}250 antibodies such as 2B2 and 30H9 (see Fig. 2 for map of the epitopes of the different monoclonal antibodies used here). **b**, A western blot containing Δ N250 and immunopurified TAFs (as a positive control) was probed with radiolabelled dTBP. Endogenous dTAF_{II}250 specifically bind radiolabelled dTBP indistinguishably, indicating that Δ N-250 is sufficient for the dTBP interaction. **c**, Immunopurification of Δ N250 from a Q-Sepharose fraction of a Sf9 cell extract infected with recombinant baculovirus. After elution of Δ N250 from the monoclonal antibody 2B2 in the presence of either 0.5 or 1.0 M guanidine hydrochloride-containing buffer, Δ N250 is pure as judged by silverstaining. IP, Immunoprecipitation. **METHODS.** The cDNA insert of λ D1 was inserted into the *Eco*RI site of baculovirus-expression plasmid pVL1393 (Pharmingen). The resulting construct was co-transfected with BaculoGold viral DNA (Pharmingen) into Sf9 cells. After 3 days cells were collected and expression of the Δ N250 protein was monitored by western blotting using the anti-dTAF_{II}-250K monoclonal antibody 2B2. The recombinant virus-containing supernatant was used to



infect large scale cultures of Sf9 cells. We typically prepared whole-cell extracts from 1 litre of plate cultures of infected Sf9-cells by sonicating the cells suspended in HEMG-ND/0.1 M KCl (HEMG-ND contains 10% glycerol, 25 mM HEPES, pH 7.6, 12.5 mM MgCl₂, 0.1 mM EDTA, 0.1% NP-40, 1.5 mM DTT, 1 mM PMSF, 5 μ g ml⁻¹ leupeptin). The supernatant was partially purified (~5-fold) by chromatography over Q-sepharose (Pharmacia) with step-gradient elution (HEMG-ND containing 0.1 M, 0.2, 0.4 and 1.0 M KCl, respectively). Δ N250 eluted in the 0.4 M salt step (Q.4 fraction). After dialysis against HEMG-ND/0.1 M KCl, the extract was frozen in aliquots. Δ N250 was immunopurified to near homogeneity from the Q.4 fraction with monoclonal antibody 2B2 immobilized on protein G beads. Bound Δ N250 was then eluted from the antibodies with HEMG-ND buffer containing 0.5 M guanidine hydrochloride for 30 min on ice. The guanidine hydrochloride was removed by dialysing the eluate against HEMG-ND for 3 h at 4 °C.

Interaction of Δ N250 with dTBP and dTAF_{II}110

Having established that Δ N250 is able to bind dTBP in a manner similar to the full-length endogenous dTAF_{II}250, we next asked whether higher-order complexes could be formed between other TAFs and the Δ N250/dTBP complex. In particular, we were interested in the relation of the putative coactivator, dTAF_{II}110, to the dTBP/ Δ N250 complex. Although dTAF_{II}110 is a component of the TFIID complex, it does not appear to bind directly to dTBP (T. Hoey, unpublished result, and Fig. 5a). Therefore we tested whether Δ N250 might be able to link dTAF_{II}110 to TBP. To address this question, we incubated *in vitro*-translated ³⁵S-labelled dTAF_{II}110 protein with recombinant Δ N250 protein in the presence or absence of dTBP. These mixtures were then immunoprecipitated with monoclonal antibodies directed against dTAF_{II}250 and the resulting complexes analysed by SDS-PAGE. dTAF_{II}110 was efficiently co-immunoprecipitated with Δ N250 (Fig. 5a), indicating that these two proteins interact specifically. In fact, dTAF_{II}110 interacted with dTAF_{II}250 even in the absence of dTBP (Fig. 5a), providing evidence for a direct TAF-TAF interaction.

To demonstrate that complexes containing recombinant dTBP, dTAF_{II}110 and Δ N250 could be formed, we immunopurified a complex with antibodies directed against dTAF_{II}250 and dTBP. To assemble this partial TFIID complex, purified recombinant dTBP and a partially fractionated extract containing recombinant dTAF_{II}110 were incubated with Δ N250 protein immobilized on protein G beads. After extensive washes, the assembled protein complex was eluted with guanidine hydrochloride and dialysed to renature the purified protein complex. Analysis by SDS-PAGE and silver staining showed that all three recombinant proteins, Δ N250, dTAF_{II}110 and dTBP could be efficiently immunopurified with anti-dTAF_{II}250 antibodies (Fig. 5b). These experiments, however, do not provide evidence that a triple complex containing Δ N250, dTBP and dTAF_{II}110 can form in the presence of these purified recombinant proteins alone. To demonstrate the formation of such a triple complex and to ascertain that purified Δ N250, dTBP and dTAF_{II}110 are sufficient to form a partial TFIID complex, we subjected these renatured purified proteins to re-immunopurification using antibodies directed against dTBP (Fig. 5b). A silver-stained SDS-

PAGE of this purified complex reveals that all three proteins reassemble, confirming the integrity and homogeneity of our partially assembled dTAF_{II}250 complex. The identity of each of these species in the triple complex was also confirmed by western blot analysis using anti-dTBP, dTAF_{II}110 and dTAF_{II}250 antibodies (data not shown). Our results show that a stable partial TFIID complex containing dTBP, TAF_{II}110 and TAF_{II}250 can be formed with recombinant proteins, and that Δ N250 can link a target of Sp1 activator, TAF_{II}110, to dTBP.

Triple complex can support Sp1 activation *in vitro*

Having assembled a triple complex containing TBP, dTAF_{II}110 and Δ N250, it was of interest to test whether this partial TFIID complex could mediate Sp1 activation. We therefore purified the complete TFIID complex, the triple complex, or a minimal complex containing only TBP and Δ N250. These antibody-affinity-purified proteins were assayed for *in vitro* transcription in the presence or absence of the purified activator protein, Sp1. As expected, transcription of a DNA template containing Sp1 sites was dramatically enhanced by purified Sp1 in reconstitution reactions containing *Drosophila* RNA pol II dTFIIB, E, F, and so on, and the immunopurified TFIID complex (Fig. 5c). By contrast, no activation by Sp1 was detected when RNA pol II and basal factors were reconstituted with a preparation of dTBP and Δ N250 instead of TFIID. In the presence of the purified triple complex containing dTAF_{II}110, however, we observed a modest but reproducible activation of transcription by Sp1. These results suggest that the triple complex consisting of recombinant dTBP, Δ N250 and dTAF_{II}110 is at least partially functional for mediating activation, presumably by serving as a coactivator complex that can interact directly with Sp1. Sp1 may also interact with other targets in the TFIID complex because full activation was not reconstituted with this partial complex.

Discussion

Since the original discovery that TFIID is composed of the TATA-binding protein, TBP, and at least seven TBP-associated factors (TAFs)^{8,10,24}, much interest has been focused on the function of these novel transcription factors, their potential role in mediating regulation, and their interaction with TBP. The

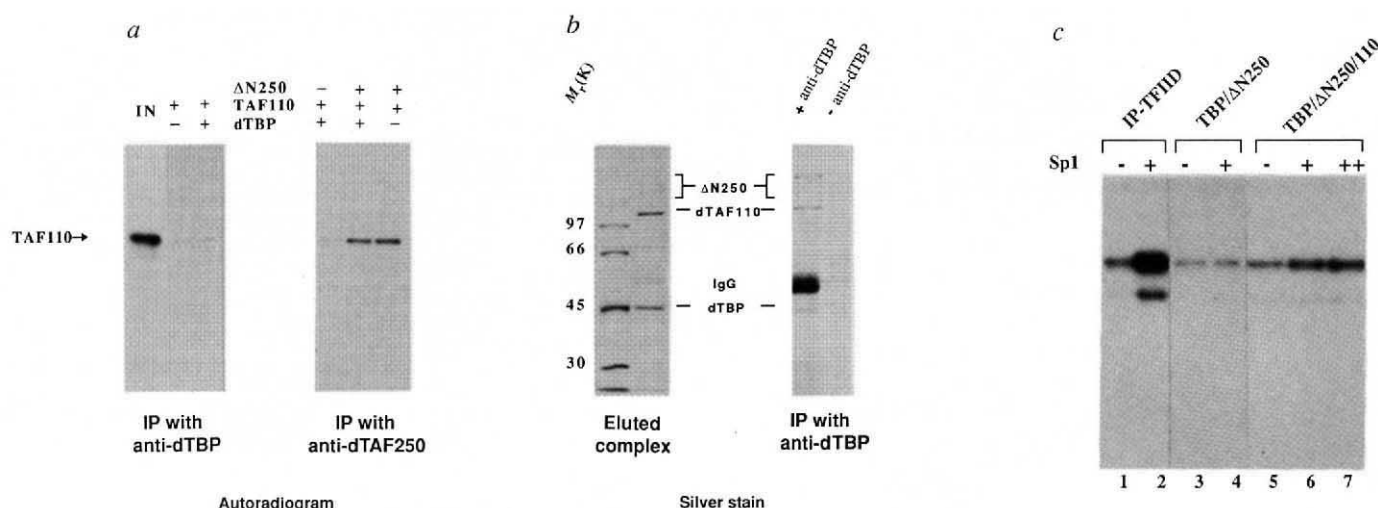


FIG. 5 *In vitro* assembly and functional analysis of a complex containing recombinant dTBP, dTAF₁₁₀ and ΔN250. **a**, Left panel: ³⁵S-labelled dTAF₁₁₀ was incubated with protein G beads preloaded with anti-TBP antibodies in the presence or absence of recombinant dTBP. dTAF₁₁₀ fails to bind to the beads in either case, indicating that dTAF₁₁₀ and dTBP do not form stable protein-protein contacts. IN, Input; IP, immunoprecipitation. Right panel: protein G beads (preloaded with anti-dTAF₁₁₀250 monoclonal antibody 30H9) were incubated with various combinations of ΔN250, ³⁵S-labelled dTAF₁₁₀ and TBP. In the presence of ΔN250 labelled dTAF₁₁₀ binds to the beads, indicating a specific interaction between the two proteins. This interaction is not affected by the presence of TBP, suggesting that dTAF₁₁₀ and dTBP occupy different binding sites on ΔN-250. **b**, Silver-stained gel of the assembled partial complex. ΔN250 was immobilized on protein G beads coated with anti-dTAF₁₁₀250 monoclonal antibody 2B2. After addition of Sf9-cell extracts containing recombinant dTAF₁₁₀ and dTBP, the complex was allowed to assemble for 45 min on ice. The assembled proteins were then eluted from the beads with buffer containing 0.5 M guanidine-HCl (left panel). After removal of the denaturant, the complex was immunoprecipitated with anti-dTBP antibody (right panel). dTBP, dTAF₁₁₀ and ΔN250 co-purify in (near)-stoichiometric quantities, proving the presence of a triple complex. **c**, Activation by Sp1 with a partial TFIID complex. All of the reactions contained recombinant *Drosophila* TBP overproduced in a baculovirus expression system and purified to homogeneity. Lanes 2, 4, 6 and 7 contain Sp1 purified to homogeneity from HeLa cells infected with vaccinia virus which expresses recombinant Sp1. Lanes 1 and 2 were supplemented with antibody-affinity purified TFIID (ref. 9). Lanes 3 and 4 were supplemented with purified

recombinant ΔN250. Lanes 5, 6 and 7 were supplemented with purified recombinant ΔN250 and dTAF₁₁₀.

METHODS. Production of recombinant dTBP and dTAF₁₁₀ in the baculovirus system: the construction of a dTAF₁₁₀ baculovirus expression construct was as described in ref. 9. Whole-cell extracts were prepared from infected Sf9 cells as described in Fig. 4. The extract was partially fractionated by passing over HEMG-ND-equilibrated Q- and S-Sepharose (Pharmacia) columns. dTAF₁₁₀ was present in the flow-through fractions of both columns. The final purity was 5% of total protein. A baculovirus-expression construct for dTBP was constructed by subcloning the entire cDNA insert of clone pdTFIID (ref. 29) into the *Eco*RI site of pVL1393 (Pharmingen). Cotransfection, amplification of the virus stock and preparation of whole cell extracts was carried out as already described. The resulting extract was purified by step-gradient chromatography on an S-Sepharose column as described for ΔN250. dTBP eluted in the 0.4 M KCl-containing fraction and was dialysed against HEMG-ND/0.1 M KCl. The purity of dTBP in the S.4 fraction was >90%. Co-immunoprecipitation studies: protein G-beads were preloaded with monoclonal antibodies and incubated with various fractions prepared from recombinant baculovirus-infected cell extracts or ³⁵S-labelled dTAF₁₁₀ prepared by *in vitro* transcription/translation. After 45 min on ice (with occasional slow vortexing to keep the beads suspended), unbound protein was removed with several washes of HEMG-ND. For some experiments this process was repeated several times with dTBP or various dTAF-containing Sf9 cell extracts to assemble multiprotein complexes from recombinant proteins. Reconstituted *in vitro* transcription with purified *Drosophila* RNA pol II and fractionated basal factors was as described⁸.

importance of TAFs was most clearly demonstrated by studies which showed that one or more of these factors serve as coactivators that are essential for activation by sequence-specific DNA-binding transcription factors, such as Sp1 and CTF^{8,10}. The situation in yeast is less clear, although TBP in yeast may also be associated with TAFs, albeit less avidly (refs 25, 26, and R. Young, personal communication).

What are the functions of TAFs in the TFIID complex, and what is their relationship to TBP and to each other? Initially, our only means of isolating these TBP-TAF complexes was to use antibodies directed against TBP and thus we naturally assumed that TBP was the 'core' subunit of the TFIID complex. TBP, however, is a relatively small protein with limited interfaces for interacting with DNA, other basal factors, and TAFs. Our studies also suggested that TAF₁₁₀s interacted predominantly with the C-terminal conserved domain of TBP, thus further limiting the number of potential interfaces. We therefore suspected that probably only one or a few of the TAFs actually contacted TBP directly. These TBP-bound TAFs might in turn serve as intermediaries that would allow the association of other TAFs into the TFIID complex. The need for such intermediary TAFs became apparent when we cloned and expressed the first TAF and found that although dTAF₁₁₀ fitted the functional profile of a coactivator by its ability to interact with Sp1, dTAF₁₁₀

did not bind directly to dTBP⁹. We therefore attempted to identify TAFs that can interact directly with TBP independently of other TAFs and found that the largest subunit of the TBP complex, dTAF₁₁₀, bound directly to dTBP under these conditions. Similar studies with human TBP and TAFs have also been reported^{19,27}. Thus, at least dTAF₁₁₀ could interact directly with dTBP, although it was possible that other TAFs also bind to dTBP but could not be detected by our assays.

The cloning and expression of a C-terminal truncated version of dTAF₁₁₀, ΔN250, has allowed us to assemble for the first time a multiprotein complex which physically links the TATA-binding protein dTBP with dTAF₁₁₀, a previously documented potential coactivator for the transcription factor, Sp1 (Fig. 6a). Such a partial complex combines at least two known properties of the TFIID complex: sequence-specific binding to the TATA box, and the presence of a putative coactivator which may mediate activation by sequence-specific DNA-binding proteins. Our findings also demonstrate at least one direct TAF-TAF interaction, as dTAF₁₁₀ can bind dTAF₁₁₀ even in the absence of dTBP. *In vitro* transcription indicates that a triple complex containing TBP, ΔN250 and dTAF₁₁₀ is capable of supporting at least modest activation by Sp1 when reconstituted with RNA pol II and the other basal factors. As expected, dTAF₁₁₀ seems to be required for Sp1 activation *in vitro* and

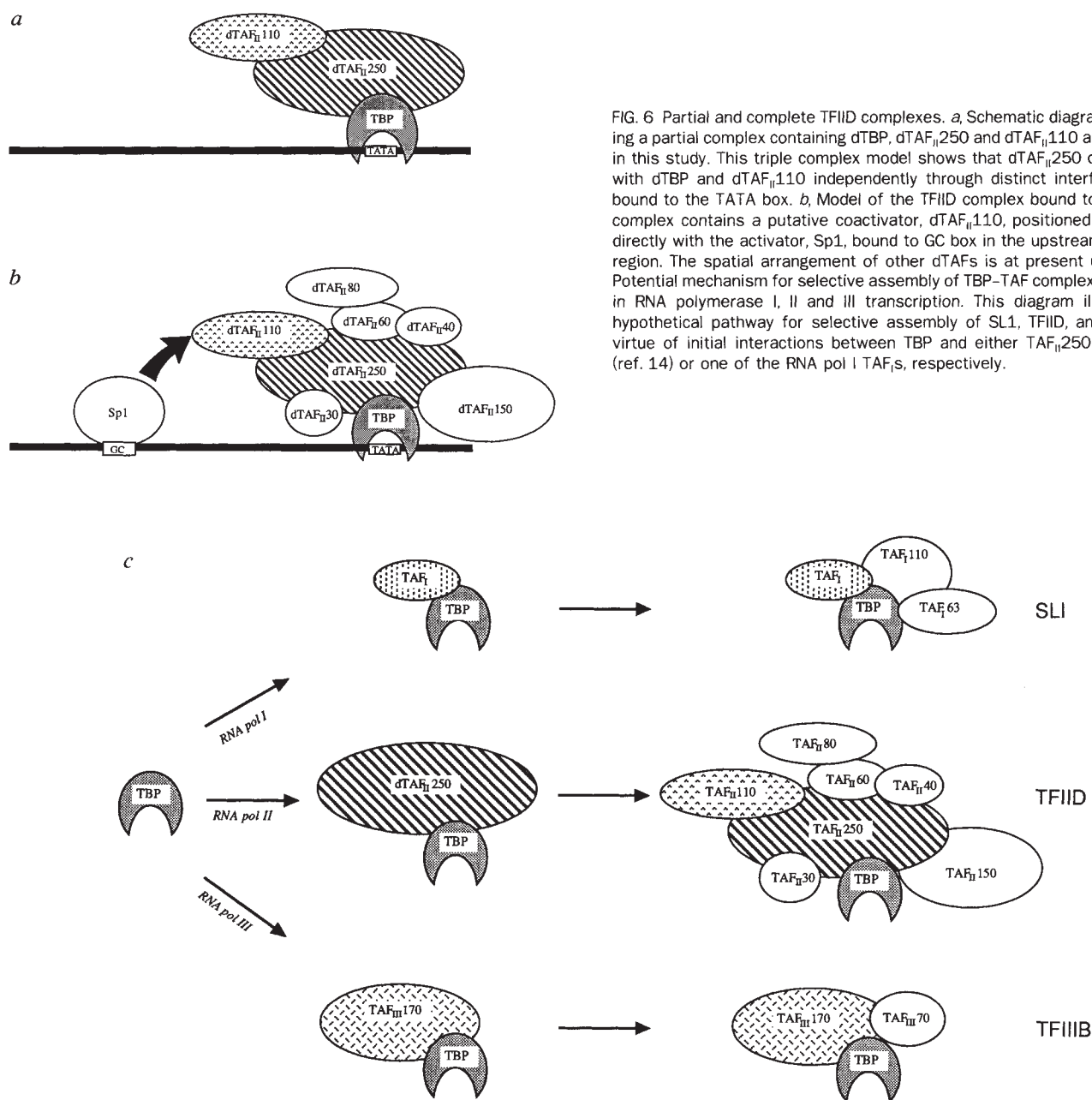


FIG. 6 Partial and complete TFIID complexes. *a*, Schematic diagram illustrating a partial complex containing dTBP, dTAF_{II}250 and dTAF_{II}110 as described in this study. This triple complex model shows that dTAF_{II}250 can interact with dTBP and dTAF_{II}110 independently through distinct interfaces when bound to the TATA box. *b*, Model of the TFIID complex bound to DNA. This complex contains a putative coactivator, dTAF_{II}110, positioned to interact directly with the activator, Sp1, bound to GC box in the upstream promoter region. The spatial arrangement of other dTAFs is at present unknown. *c*, Potential mechanism for selective assembly of TBP-TAF complexes involved in RNA polymerase I, II and III transcription. This diagram illustrates a hypothetical pathway for selective assembly of SL1, TFIID, and TFIIB, by virtue of initial interactions between TBP and either TAF_{II}250, TAF_{III}170 (ref. 14) or one of the RNA pol I TAFs, respectively.

dTAF_{II}250 assembles a complex containing TBP and dTAF_{II}110. But Sp1-dependent action with the complete TFIID complex was significantly more potent than with the triple complex. Thus, it is likely that activators such as Sp1, which contain multiple activation domains, may interact with more than one target or component of the TBP-TAF complex. This partial complex of course still lacks other TAFs which are present in the intact TFIID complex (Fig. 6*b*). It is likely, therefore, that more TAFs will interact with the large subunit, dTAF_{II}250, and that some of these other subunits may also be necessary to obtain a fully functional complex with coactivator activity. It should also be noted that Δ N250 is a truncated version of dTAF_{II}250 lacking some N-terminal sequences, which may be important for other functions not detected by our assays. *In vitro* assembly of partial complexes with purified recombinant proteins such as those described here should eventually allow us to investigate in greater detail the contribution of individual TAFs to the architecture and function of the TFIID complex.

An important finding with implications for general mechanisms of transcriptional control in eukaryotes was the discovery that TBP and unique sets of TAFs constitute integral components of initiation complexes governing not only messenger RNA transcription by RNA polymerase II, but also transcription of ribosomal RNA by RNA polymerase I (ref. 13), and more recently, transfer RNA synthesis by RNA polymerase III (refs 1, 14–17). Thus, this unusual arrangement of distinct sets of TAFs associated with the universal transcription factor TBP probably underlies a common mechanism of transcriptional control in higher eukaryotes. From our results, it has become clear that the interaction between dTBP and dTAF_{II}250 almost certainly represents a control point for regulation of transcription in eukaryotic cells. What mechanism could be responsible for channelling TBP into various basal transcription complexes such as SL1, TFIID, TFIIB? As dTAF_{II}250 can form protein-protein complexes directly with dTBP as well as with other TAFs, we suggest that the interaction of this large subunit with

TBP could determine the amount of functional TFIID complex available for RNA pol II transcription within the cell (Fig. 6c). By analogy, we propose that specific pol I and pol III TAFs could serve similar functions to nucleate the assembly of SL1 and TFIIB, respectively. □

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Spectrally resolved eclipse maps of the accretion disk in UX Ursae Majoris

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ACCRETION disks play an important role in many astrophysical environments, such as active galactic nuclei, protostellar systems, X-ray binaries and cataclysmic variables. The lack of spatially resolved information, however, has meant that theoretical models for accretion disks are in general poorly constrained by observations. Here we use the shape of the light curves from an eclipsing cataclysmic variable, UX Ursae Majoris, to reconstruct the spectral energy distribution (in the range 3,600–10,000 Å) across the face of an accretion disk. The spectral resolution is sufficient to reveal both the radial dependence of absorption and emission line features within the disk, and the spectral details of the bright spot formed at the point where the accretion stream from the secondary star collides with the disk. Such detailed reconstructions of accretion-disk spectra should help to bridge the gap between observations and theoretical models.

The nova-like cataclysmic variable UX Ursae Majoris (UMa) is an eclipsing interacting binary system with a relatively stable, high mass-transfer rate. On the nights of 1992 June 4, 6 and 7 we observed UX UMa under excellent conditions with the Faint-Object Spectrograph (FOS) on the 2.5-m Isaac Newton Telescope (INT) located at the Observatorio del Roque de los Muchachos, La Palma. The FOS provides low-resolution spectra covering the wavelength range from the near ultraviolet to the near infrared in two spectral orders, and is equipped with a GEC charge-coupled device (CCD) detector. The first order of the grism (grating plus prism) gives a dispersion of 10.7 Å per pixel and the second order gives 5.4 Å per pixel¹.

We took a series of spectra during the eclipse, with an exposure time of 45 s each, yielding an effective time resolution, including overhead for reading out the CCD, of ~90 s (or 0.0053 of the

4.72-hour orbital period). A wide spectrograph slit was used to minimize (variable) slit losses, but at the cost of having the spectral resolution influenced by the size of the stellar image. The observations of UX UMa were regularly interrupted for measurements of a local comparison star, which were used to correct the spectra of UX UMa for (wavelength-dependent) variations in atmospheric extinction. We employed standard data-reduction techniques (correcting for the electronic bias level and pixel-to-pixel sensitivity differences, sky subtraction and extracting the spectrum by summing over the stellar profile). The wavelength scale was calibrated using arc lamp exposures. The flux scale was established through observations of a flux standard star; the absolute flux scale becomes less reliable towards the highest wavelengths because of low count rates and the presence of strong atmospheric absorption bands.

Figure 1 displays representative spectra of UX UMa out of eclipse, and at mid-eclipse. At mid-eclipse the continuum light drops substantially whereas the flux in the Balmer emission lines (except H α) increases. This indicates that the centre of the accretion disk (including the white dwarf), which is almost completely obscured at mid-eclipse, must have absorption lines that nearly cancel the emission features from the outer disk out of eclipse. The absence of an eclipse of the emission line feature does not automatically imply that the line is formed in a vertically extended volume (for example a wind coming from the disk) as has been suggested².

The individual spectra were binned into 50 Å intervals with the exception of the major spectral lines of hydrogen and helium, which were binned into 3,000 km s⁻¹ wide intervals. For each of the 110 spectral intervals a light curve was constructed by summing the flux in each bin and folding the resulting data on the orbital period³, with phase zero centred on primary eclipse (using the recent eclipse timings in ref. 4). The brightness of UX UMa differs slightly over the three nights, probably because of the intrinsic variability of UX UMa, or variability of the comparison star. These small differences were taken out by scaling the light curve onto a common absolute flux outside eclipse.

For the analysis of the eclipse light curves we employed the maximum-entropy eclipse-mapping technique^{4,5}. The algorithm used assumes a flat, thin disk which lies in the orbital plane. Hence, any orbital variation, apart from the eclipse itself, cannot be accounted for. In the light curve of UX UMa there is a small hump just before eclipse. This hump has been taken out by fitting a cubic spline to the light curve outside eclipse, dividing the intensities by the spline fit, and multiplying by the value of

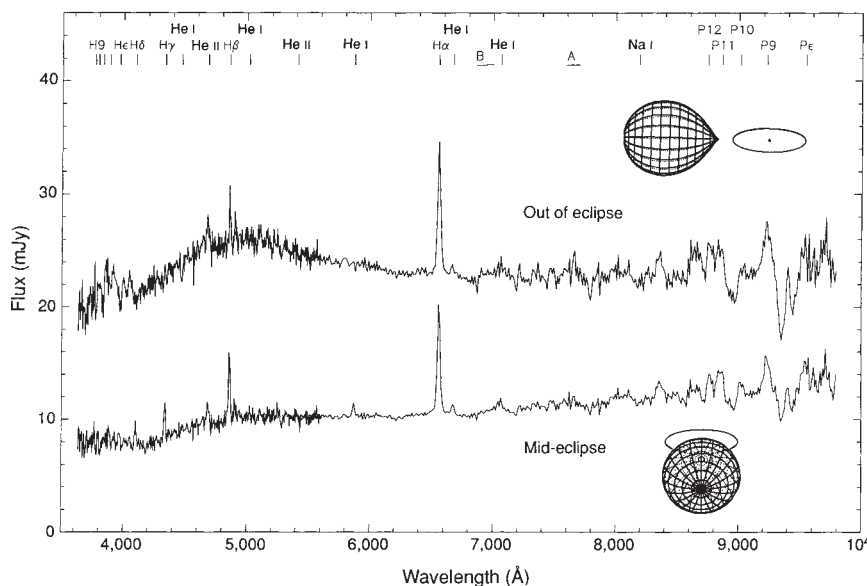
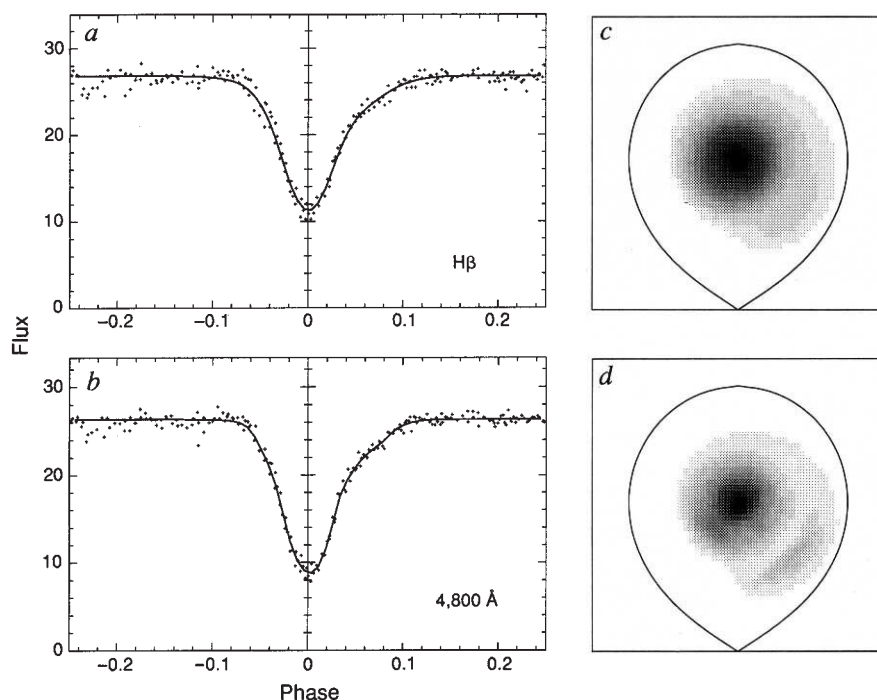


FIG. 2 Narrow-band light curves of UX UMa, centred at the $H\beta$ line (a) and at 4,800 Å in the nearby continuum (b), in mJy. The continuous lines represent the fits to the light curves. The corresponding disk images are presented as c and d. The Roche lobe, with the L1 point at the centre of the lower boundary, is plotted for reference.



the spline at zero phase. This procedure removes any gross variations outside eclipse but hardly affects the eclipse shape itself.

Two representative light curves are shown in Fig. 2, for the $H\beta$ line and a nearby continuum band at 4,800 Å. The deep eclipse of the central disk and the slow egress due to the bright spot are readily seen.

All light curves were fitted independently with our eclipse-mapping algorithm, which minimizes the structure of the disk brightness distribution relative to some smooth default map. Usually this default map is an azimuthally smeared version of the actual solution. Other definitions of the default map may however be used to steer the solution towards a specific shape, within the constraints set by the observations and their uncertainties⁵. The orbital inclination of UX UMa—71 degrees—is relatively small for an eclipsing system, and as a result the far side of the disk remains unocculted at all phases. Because the eclipse does not contain direct information on this part of the disk, full azimuthal smearing of the default map results in relatively high intensities in that unocculted section, at the same radial distance from the disk centre as the bright spot. To alleviate this problem we limited the azimuthal smearing in the default map by using a gaussian profile with a width of 30°.

Statistical uncertainties in the disk brightness distribution have been determined by fitting each light curve several times, after the observed data had been subjected to random fluctuations corresponding to their uncertainties. These trial fits yield the uncertainty for each pixel on the maps.

There are sources of systematic errors apart from the statistical uncertainties, such as the presence of the secondary star, the assumption that the disk is thin and flat, and possible errors in the adopted orbital inclination, conjunction phase and mass ratio. Light from the secondary star, which has been neglected in our analysis, is expected to contribute about 12% to the total light at 8,000 Å, but is negligible in the blue part of the spectrum⁴. Hence, although our approximation for the secondary star being 'black' induces systematic errors, its effect is only small. The impact of the assumptions will be discussed in more detail elsewhere.

The fits to the light curves in Fig. 2 are shown as solid lines. Similar fits have been obtained for light curves at each wavelength interval, producing a series of narrow-band images

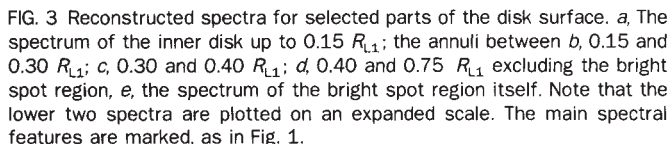
of the accretion disk. Combining these images rebuilds the integrated disk spectrum, but any part of the disk surface can be isolated and its spectrum reconstructed. In Fig. 3 we present the spectrum of the inner disk surface up to 0.15 R_{L1} (R_{L1} is the distance between the white dwarf and inner Lagrange point L1), and the spectra for the disk annuli between 0.15 and 0.30 R_{L1} , and 0.30 and 0.40 R_{L1} . The outermost ring from 0.40 to 0.75 R_{L1} has been examined in two sections: the bright spot region (covering one quadrant) and the remaining outer disk. The error bars reflect the variance on the fluxes over the averaged region.

The spectra in Fig. 3 show that the inner disk spectrum peaks in the blue at ~4,000 Å and that further out in the disk the spectra become progressively redder. This reflects the decrease in the surface temperature of the optically thick disk from roughly 17,000 K in the inner zone to roughly 6,000 K in the outer disk. Another clear distinction between the inner and outer disk spectra is seen in the Balmer lines, which are in absorption in the inner disk (with the exception of $H\alpha$), and in emission in the outer disk regions.

The spectrum of the bright spot region is relatively blue and appears to have (weak) Balmer absorption lines, in contrast to the outer disk spectrum at the same radial distance from the centre of the disk, which exhibits Balmer emission lines. This naturally explains the absence of an s-wave in the trail spectrum of the Balmer emission lines², which in several other cataclysmic variables has been associated with radiation from the bright spot region (see for example ref. 6).

Our current model assumes that all light is coming from the disk, but there are indications that in other nova-like systems part of the line radiation is coming from an extended region above the disk⁷. Although there is no reason to invoke emission line radiation from above the disk to understand the present data, we cannot exclude the possibility that such an additional light source is interpreted here as line radiation coming from the disk.

Qualitatively, the spatially resolved spectra presented in Fig. 3 agree with simple models for accretion disk spectra^{8,9} which predict an optically thick inner disk and an optically thin outer disk. A quantitative comparison of the disk spectra, however requires a range of spectral models tailored to the specific case of UX UMa, because disk spectra are sensitive to details such



as the orbital inclination and the mass-transfer rate¹⁰. These parameters have been determined with reasonable accuracy for UX UMa, but matching models have not been published yet. Also, comparison of our results with spectral model calculations which involve the spectral variations across the disk is not possible with current published models. This is because researchers have concentrated on modelling disk integrated flux distributions as there is a lack of observed spatially resolved spectra. Our results will allow models of the local vertical structure of accretion disks to be tested for the first time.

The eclipse-mapping technique can be used to analyse time-resolved spectrophotometric data (rather than just broad-band photometric light curves) to reveal detailed spectral characteristics across the accretion disk surface. This should allow us to compare radiative transfer calculations with observations in much more detail, and bring us closer to understanding the physical conditions that exist in accretion disks. It is possible that (for suitable emission-line objects) the spatially resolved line brightness information deduced from spectral eclipse mapping, combined with the line velocity information deduced from Doppler tomography¹¹, will allow us to study the velocity fields in accretion disks. □

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THE discovery¹ and bulk synthesis² of carbon nanotubes has stimulated great interest. It has been suggested that these structures may have useful electronic³⁻⁵ and mechanical⁶ properties, and these might be modified by introducing foreign materials into the nanotubes. But the tubes are invariably capped at the ends. Ajayan and Iijima⁷ have succeeded in drawing molten material (lead or one of its compounds) into the tubes by heating them in the presence of lead and oxygen; less than 1% of the tubes in the sample studied could be filled in this way. Here we report that heating in carbon dioxide gas can result in the partial or complete destruction of the tube caps and stripping of the outer layers to produce thinner tubes. In some cases, we have thinned the extremity of tubes to a single layer. The opened tubes can be regarded as nanoscale test-tubes for adsorption of other molecules, and this controlled method of thinning may allow studies of the properties of single tubes.

The carbon nanotubes were prepared under conditions that are essentially the same as those used for C₆₀ production^{8,9}. An arc was struck between two electrolytic-grade graphite rods (8 mm outer diameter, 15 cm in length, purity >99%) in 100 torr of helium using a d.c. voltage of 30 V and a current of 180–200 A. Under these conditions the anodic graphite rod was found to evaporate and the cathodic rod increased in length. Roughly 20–30% of the carbon vaporized from the anode distills onto the cathode, resulting in a gain in length of 3–4 cm. The carbon material containing the nanotubes is found at the black central core of the cathodic rod. Heat treatment of this material was carried out in a silica tube between silica wool plugs. The material was heated under a flow of 20 ml min⁻¹ CO₂ at 850 °C for 5 hours. A weight loss of 10% was recorded. We measured the surface area of the material by the Brunauer, Emmett and Teller (BET) method¹⁰ using N₂ at -196 °C. Samples were prepared for high-resolution transmission electron microscopy by dispersion of the material in chloroform and deposition onto a holey carbon film. The microscope used was a JEOL 4000FX instrument, operated at optimum defocus with an accelerating voltage of 100 kV.

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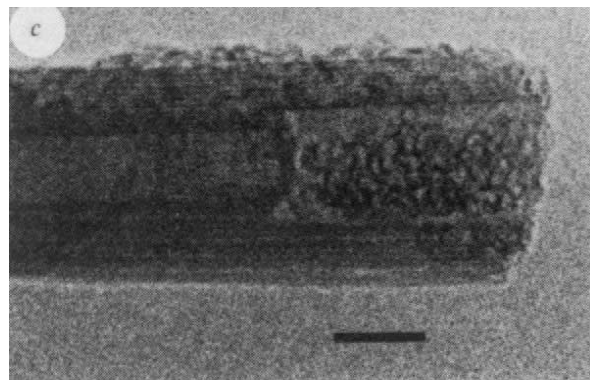
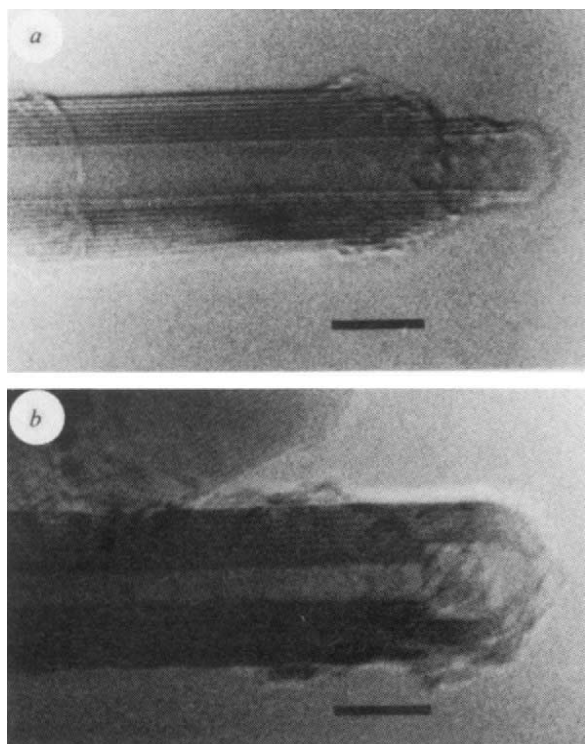


FIG. 1 *a*, Carbon-dioxide-treated nanotube in which some outer graphitic layers of the cap have been stripped off and a thinner inner tube extends beyond the region. *b*, Partially opened cap of a carbon-dioxide-treated nanotube with some terminated fringes inside the tube. *c*, A completely uncapped nanotube in a carbon dioxide-treated sample with some amorphous material inside the tube. In each image the scale bar is 50 Å.

Detailed examination of the freshly prepared soot showed that the nanotubes were invariably capped and that the caps could take a wide variety of morphologies^{11–13}. An extensive survey of the nanotubes revealed that all the tubes are seamless with no broken caps, and the continuity of lattice fringes could be traced. The internal diameters varied from ~8 to 100 Å.

After treating the material with carbon dioxide, we found that a small but significant proportion (about 5–10%) of the nanotubes had suffered some corrosion in the cap region. Figure 1*a–c* shows typical examples. It can be seen in Fig. 1*a* that graphitic layers have been stripped off near the tips of some nanotubes and there is a short protruding inner tube, of a type not observed in the samples before treatment with carbon

dioxide. All of the corrosion we have observed occurs near the tip and there is no observation of any pitting, or necking, of nanotubes away from the caps. Figure 1*b* shows a partially destroyed graphitic curved cap with some terminated fringes inside the tube. Figure 1*c* shows a nanotube in which the cap has been completely destroyed, exposing the internal cavity. It seems that some amorphous material has entered the tube above an internal cap. A lower-magnification micrograph of a nanotube with a wide internal cavity (Fig. 2) shows that the graphitic layers of the cap have been selectively removed, although there appears to be a single remaining layer across the opening. Figure 3 shows an example of a nanotube with the outer graphitic layers stripped off, apparently leaving a single layer tube near

FIG. 2 Lower-magnification image of a carbon dioxide-treated nanotube with an almost completely destroyed cap. The scale bar is 50 Å.

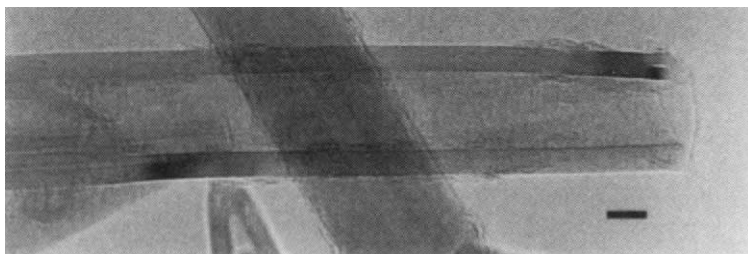
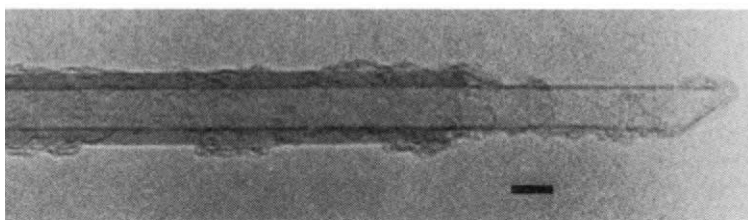
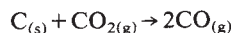


FIG. 3 Carbon-dioxide-thinned nanotube, the tip of which appears to have only a single graphitic layer. The scale bar is 50 Å.



the tip. A nanotube that is one graphitic layer thick has not been observed before, although the properties of such tubes have been the subject of much speculation¹³. Preparing single-layer nanotubes by the selective oxidation method described here may enable these predictions to be tested. We also measured the surface areas of the bulk samples before and after carbon dioxide treatments and found that surface areas increased from 21.0 to 31.7 m² g⁻¹.

In our experiments we use the mild oxidizing agent carbon dioxide, and the chemical reaction which occurs is



This reverse Boudouard reaction is thermodynamically favour-

able with the equilibrium constant K_p of 1.76 at our reaction temperature¹⁴, although the equilibrium constant of entirely closed graphite tubes is likely to be slightly different from other types of carbon.

Our observations indicate that the more reactive outer carbon layers at the tip of the cap selectively react with carbon dioxide and are subsequently stripped off. The selective attack of the curved part of a cap reflects greater reactivity which may be due to greater strain and the presence of pentagonal rings⁷. Destruction of the cap exposes the terminated cylinder layers which can then be further eroded to form thinner tubes. The observed increase in surface area may be explained by a combination of a reduction in the average tube diameter and the removal of caps from some of the nanotubes. □

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Opening carbon nanotubes with oxygen and implications for filling

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CAPPED hollow carbon nanotubes^{1,2} can be modified into nanocomposite fibres by simultaneous opening of the caps (by heating in the presence of air and lead metal) and filling of the interior with an inorganic phase³. To generalize this approach, greater understanding is needed of the reaction mechanism between the tube caps and oxygen. Here we report that the oxidation of carbon nanotubes in air for short durations above about 700 °C results in the etching away of the tube caps and the thinning of tubes through layer-by-layer peeling of the outer layers, starting from the cap region. The oxidation reaction follows an Arrhenius-

type relation with an activation energy barrier of about 225 kJ mol⁻¹ in air. Heating of closed nanotubes with an oxide (Pb₃O₄) in an inert atmosphere lowers the activation barrier for the reaction and opening of the tubes occurs at lower temperatures. Contrary to intuition, however, open tubes are much more difficult to fill with inorganic materials than in the one-step filling of tubes reported previously³. But various other experiments might be possible in the inner nano-cavities of the open tubes such as studies of catalysis and of low-dimensional chemistry and physics.

The experiments were done with carbon nanotubes produced by the arc-discharge method described previously². Weighed amounts of the carbon nanotube samples were heated in air in an open quartz vessel. The samples started to show substantial loss of weight once they were heated above 700 °C. At ~850 °C the entire sample disappeared when heated for ~15 minutes. Figure 1 shows the plots corresponding to the weight-loss measurements at various temperatures for a given time period. As seen in the case of high-grade carbon fibres⁴, carbon nanotubes show a fairly high resistance to oxidation (negligible weight loss) up to some critical temperature (700 °C in air),

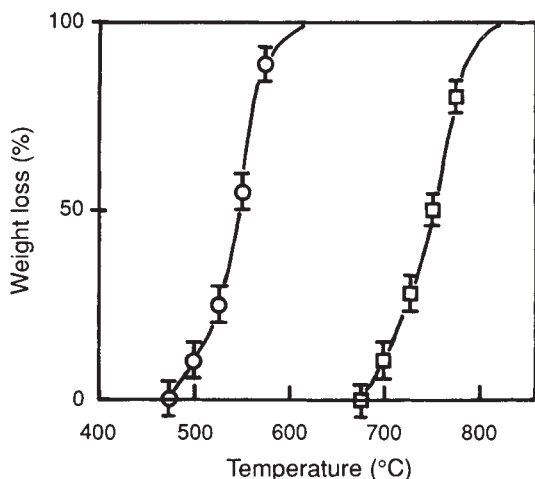


FIG. 1 Percentage weight loss of C₆₀ (circles) and nanotube/nanoparticle mixture (squares) as a function of temperature of baking in air for a given time period (15 minutes).

above which oxidation proceeds rapidly. The oxidation process follows the Arrhenius relation for temperature dependence from which we find an activation energy barrier of $\sim 225 \text{ kJ mol}^{-1}$ in air. C_{60} is consumed in air at much lower temperatures ($\sim 500^\circ\text{C}$) than the carbon nanotubes, as can be seen in Fig. 1. Despite the wide distribution in shapes and sizes of nanotubes, the oxidation curves are smooth (no steps or double peaks) over the temperature range we measured, much like that of pure C_{60} .

The state of the oxidized samples was surveyed using a transmission electron microscope, revealing that the oxidation reaction occurs preferentially at the tips of the tubes, as seen from the low-magnification image of Fig. 2. More than $\sim 20\%$ of the tubes seen are completely open for a specimen heated in air at 800°C for 10 minutes. This percentage might change with the quality of the sample (average size of the nanotubes and ratio of nanoparticles) and the actual availability of oxygen to the sample. Nanoparticles are also seen to be attacked by oxygen with the removal of layers from the surface, but they seem to be much more stable than the tube caps (see Fig. 2). Even for tubes that are not completely open, peeling of the outer layers has occurred, initiating at the capped ends of the tubes (Fig. 3a). This peeling results in the thinning of the tubes layer by layer and seems to be just the reverse of the layer-by-layer growth mechanism that we have proposed⁵. An extreme example of this thinning from the cap to the bottom of tubes is shown in Fig. 3b, where a large tube has been opened at the tip and massively eroded from the edges. The opening of the tubes should also increase the total surface area of the sample by exposing the inside surfaces of the tubes. Figure 3c shows an opened tube, and it is evident that when tube caps open up, carbonaceous debris is sucked in (compare with the empty hollow in Fig. 3a).

From the observations, it is clear that the oxidation of the tube caps occurs at a temperature which is higher than the reaction temperature of C_{60} but lower than the temperature at which the graphite basal planes react and are consumed. Con-

sidering that the caps of the tubes react first, both the strain at the tip and the presence of pentagons might help to initiation of the oxidation process. It is known from the synthesis of C_{60} derivatives that the pyracylene moieties involving two pentagon rings is important for the chemical reactivity of C_{60} (ref. 6). In nanotubes, however, the pentagons are generally not close enough to form pyracylene units. Furthermore, nanoparticles oxidize much less readily although they have the same number of pentagons as nanotubes (12, by Euler's rule). The difference between nanoparticles and nanotube tips is only in the degree of curvature, and thus the strain. So although pentagons might play a role, strain must be the key factor in the onset of oxidation at the nanotube tips.

Intuitively one would expect that with the tube ends open it would be much easier to introduce low-melting-point materials and even pure metals, by heating in vacuum. But in fact it was very difficult to fill the open tubes with another material. When open tubes were heated in the presence of liquid lead in vacuum or an inert gas atmosphere, the outer peripheries of the open tube ends seem (from the contrast) to become decorated with a metallic phase, but no filling was observed (see Fig. 4a) despite repeated attempts under different conditions.

Previous experiments with lead metal in air³ indicated that opening of tubes could occur at much lower temperatures ($\sim 400^\circ\text{C}$). As we have shown here that nanotubes open only at much higher temperatures, the reactions in the case of Pb must have been catalysed either by lead particles or some form of lead oxide. To verify this we heated carbon nanotubes with Pb_3O_4 (red lead) in an argon atmosphere above the melting point of the oxide (600°C). It was seen that a few of the tube tips opened up and were filled with lead material (Fig. 4b). Powder diffraction data from the heated material suggest that red lead had been changed to the lower oxide (PbO) and pure lead metal. Again the onset of the oxidation reaction shifted to lower temperature, perhaps because of the close contact with

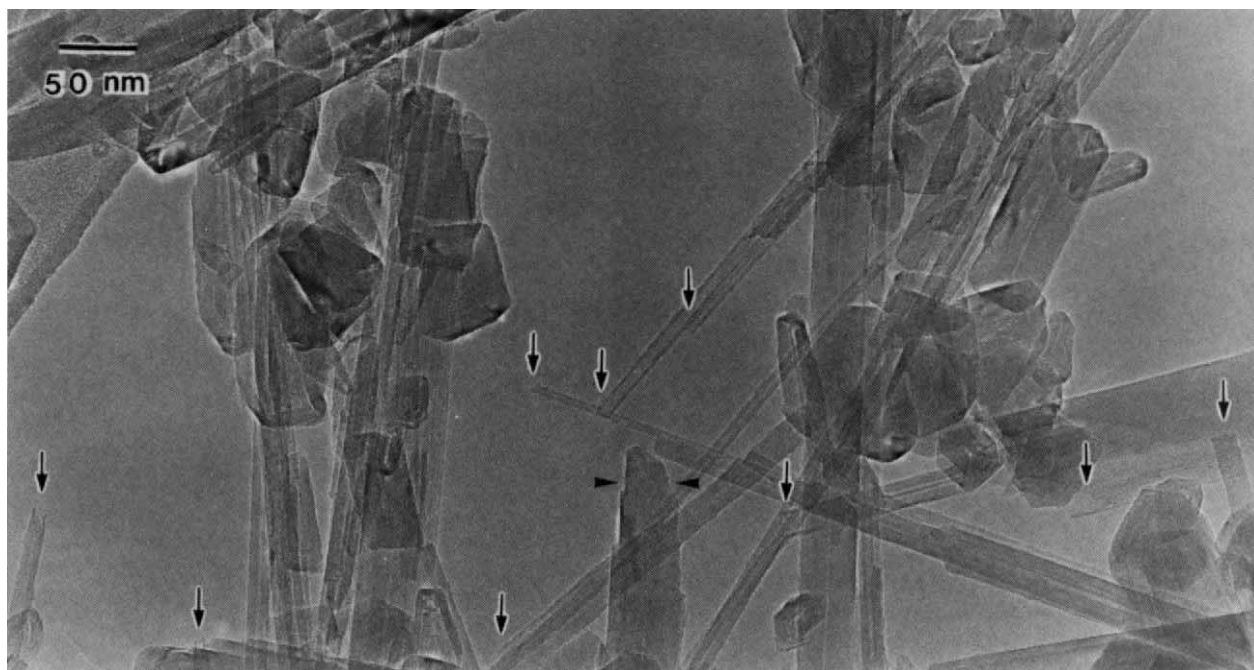


FIG. 2 Low-magnification transmission electron micrograph showing a mixture of carbon nanotubes and nanoparticles that has been heated in air at 800°C for 10 minutes. Vertical arrows show the tube tips that have become

open during annealing. Horizontal arrowheads show a large tube that is not completely open but has been eroded from the top and sides. Many large nanoparticles have survived.

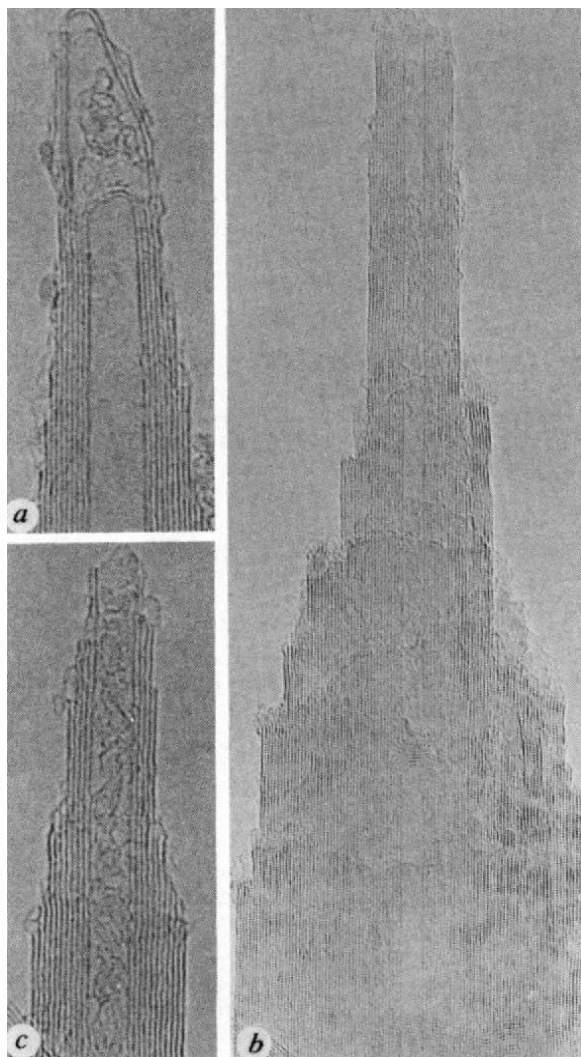


FIG. 3 High-resolution images of individual carbon nanotubes showing the structural damage that has occurred during oxidation in air. *a*, Conical tube which is not completely open but shows the removal of layers, starting from the cap region. *b*, Large tube which has been massively eroded at the edges. Shadows of the peripheries of outer, open tubes are seen clearly in the image. *c*, Opened tube showing carbonaceous debris that has been sucked into the inside hollow. Distance between fringes in the images is 0.34 nm.

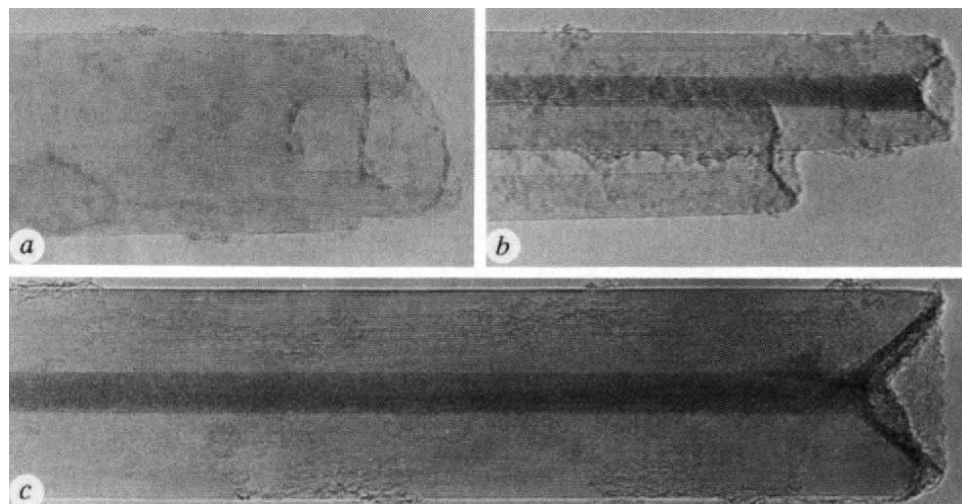
the metal oxide. We have also done experiments in pure oxygen (1 atm) but the reaction equilibrium was not shifted as much as when we used the oxide. Above $\sim 750^\circ\text{C}$, in pure oxygen, an ignition point is reached and sparks emerge from the sample.

Because the carbon nanotubes open readily in air at $\sim 800^\circ\text{C}$, it should be easy to introduce any oxides that are molten at or below this temperature. We have successfully filled the nanotubes with bismuth in the presence of oxygen by heating closed tubes with Bi metal in air at $\sim 850^\circ\text{C}$ (Fig. 4c). The phase of the filling inside seems not to correspond to pure Bi metal (on the grounds of image and contrast). The weight loss of the sample when heated with Bi metal was much lower than expected, possibly because part of the oxygen supply in air was made available for the oxidation of Bi metal. Although it seems difficult to fill the inside cavities of already open tubes with pure metals by heating in vacuum, it might be possible to introduce unreactive metals such as gold by carefully controlling the partial pressure of oxygen (shifting the oxidation reaction equilibrium to higher temperatures) during the heat treatment. It should be easier for fluids and other low-viscosity materials to enter the tubes. Preliminary results show that it is possible to introduce Pt particles prepared in solution from dissolved $\text{K}_2(\text{PtCl}_4)$ into some larger cavities of open tubes. It might be possible in future to perform catalysis or solution chemistry in the channels of open tubes and compare with experiments done outside.

It is clearly much easier to fill nanotubes in a one-step process from closed tubes than in a two-step process in which one first makes open tubes. The capillarity forces in the nanometre-sized cavities⁷ may be adversely affected by contaminants sucked in during the opening process. During the filling process, capillarity forces compete with factors such as surface tension, the metal viscosity and so on. But how are these problems overcome when the nanotubes are filled in a one-step process from closed tubes? One possibility is that the inside cavities of the closed tubes are vacuum, or near-vacuum, and that when they are opened in the presence of the metals, the latter are sucked in. The chance of He or other inert gases being present inside the cavities of fullerenes is remote, as pointed out recently⁸, and this adds weight to our argument. The meaning of vacuum in such small cavities is unclear. A simple estimate shows that only about 20 gas molecules would be found in a $1\text{-}\mu\text{m}$ -long, $10\text{ \AA}$ hollow tube at 1 atm pressure, assuming no adsorption. In such small domains the description of usual physical quantities such as pressure and surface tension is not obvious⁹.

The availability of open tubes not only leads to new physics but should also open the possibility of interesting chemical

FIG. 4 Images showing filling of tubes. Separation between two parallel fringes is 0.34 nm. *a*, Open tube that has been heated with lead in Ar gas. No filling is seen but the periphery of the open end is seen decorated to be with, probably, the metallic phase. *b*, Filling of a lead phase inside a nanotube when closed tubes are heated with red lead in Ar. *c*, Filled nanotube with a bismuth phase filling.



catalysis, one-dimensional chemistry and perhaps even chiral chemistry. Tubular structures are also found in nature, so they might be used in biomimetic systems. As fluids should be able to move freely through the open ends of the tubes, physical phenomena involving constrained equilibrium due to strong capillary effects (such as the behaviour of superfluids) may be tested in the future. □

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Continuous anchoring transition in liquid crystals

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THE search for new ways of aligning liquid crystals¹ is motivated by their potential for optical and optoelectronic device applications. Rubbing of surfaces coated with an 'aligning agent' can induce an orientational preference in adjacent liquid-crystal films. Most of these surface preparations produce strong anchoring, in which the alignment remains pinned to the surface-induced orientation. But it is expected that for weak interactions with the surface, anchoring transitions may be possible in which the liquid-crystal orientation can be changed continuously, as a function of temperature for example². Here we show that an anchoring transition can be observed for liquid crystals in contact with fluoropolymers as aligning agents. We find that the phenomenon might be general, and suggest that it provides the possibility of controlling precisely the angle of liquid-crystal alignment, and a method of producing weak anchoring.

It is well known that weak surface forces can be used to orient liquid crystals¹. A simple process of rubbing a glass plate with a piece of cloth in a given direction forces the liquid-crystal molecules to orient in the direction of rubbing. Although there continues to be some debate about the mechanism of alignment³, it is clear that at least in certain cases where the surfaces are coated with stretchable polymers, the alignment is due to the physical orientation of the polymer chains during the process of rubbing⁴. This oriented polymer then induces orientation of the molecules by pseudo-epitaxy; non-mesogenic materials such as polymers⁵ may also be aligned in this way. This type of ordering, in which the orientation of the molecules is in the plane of the substrate, is often referred to as planar or homogeneous alignment. In another type of ordering, called homeotropic alignment, the director forms an angle of 90° with the surface plane. This arrangement can be produced by coating the surface with certain surfactants.

Most aligning materials produce one type of alignment or the other and although small deviations from 0° or 90° are possible, any arbitrary value between these values is generally difficult to achieve⁶. In some systems, however, the angle that the director makes with respect to the surface plane is temperature-dependent, and therefore in principle both types of alignment can be produced as a function of temperature. Ryschenkow and Kleman⁷ were able to observe anchoring transitions of the nematic liquid crystal methoxybenzylidene butylamine (MBBA). By observing defects in these samples they were able to estimate the anchoring energy, and to show that these structural transitions were related to low anchoring energies. This observation has never been repeated or improved, and it has not been clear whether this is a unique system or whether other liquid crystals might exhibit similar behaviour.

A 0.05–2% solution of Fluorad 431 in ethanol or isopropanol was deposited on indium-tin-oxide-coated glass by a conventional spinning process, then dried at ~120 °C for varying times ranging from 30 minutes to ~24 hours. The typical thickness of the polymer layer was estimated to be 300–3,000 Å. Some plates were then rubbed to produce uniform in-plane orientation of the director, and the sample cells were made by using fibre spacers of the appropriate thickness, typically ~10 µm. This was deliberately chosen so that if the surface anchoring is weak, one should be able to observe wall defects whose width is $\sim \sqrt{hK/W}$, where K is the elastic constant, h is the sample

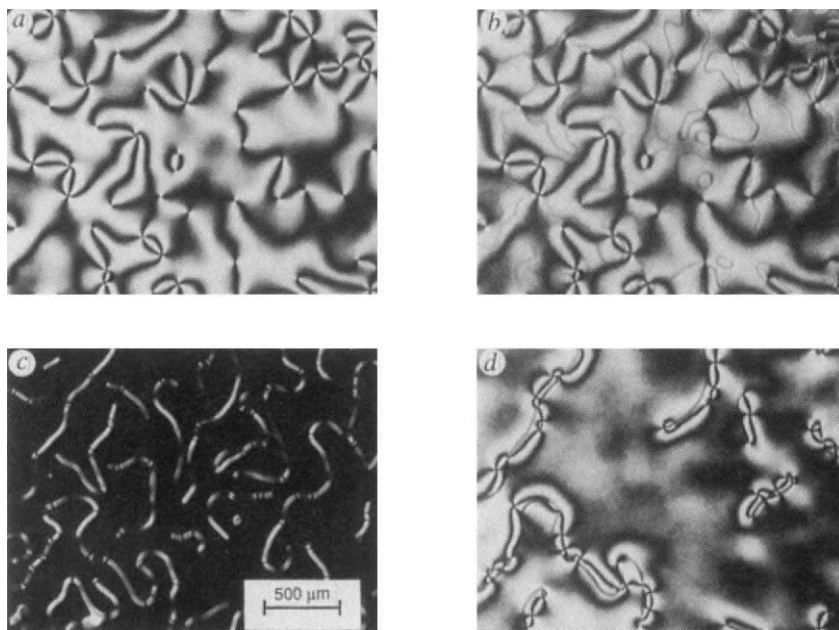


FIG. 1 Micrographs of a sample of liquid crystal at various temperatures. The sample was contained in a cell coated with fluorosurfactant and placed between cross-polarizers. The liquid crystal was E7 and the temperatures are (a) 40 °C, (b) 45 °C, (c) 55 °C and (d) 57 °C. All micrographs are at the same magnification.

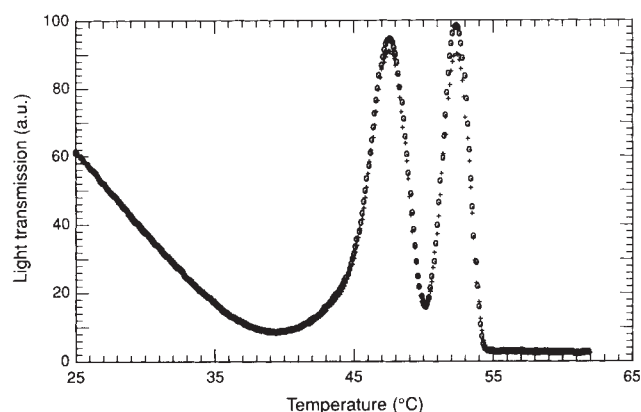
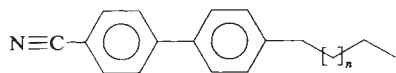


FIG. 2 Transmitted light intensity (in arbitrary units) of an oriented sample observed between cross-polarizers as a function of temperature. The two symbols represent the heating (+) and the cooling (○) cycles and show that there is no hysteresis.

thickness and W is the anchoring energy⁵. Liquid crystals such as E7, which is a mixture of cyano-biphenyls,



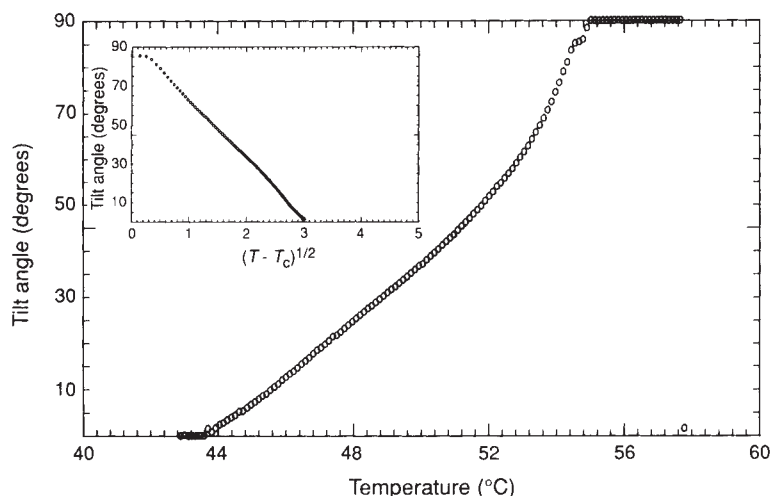
were inserted into the cell in the isotropic state in order to avoid any flow-induced alignment. During the cell-filling process, it was much more difficult to use the capillary to introduce the liquid crystal into these cells compared with those coated with a conventional aligning agent, because of the low surface tension of the fluorosurfactants. The cell was observed by a polarizing microscope in conjunction with a Mettler hot stage to control the sample temperature.

We consider a typical sample that was coated with 1% solution and baked at 120 °C for 30 min. In unoriented samples, at temperatures much below the isotropic-to-nematic phase transition, the material showed a Schlieren texture with half- and full-strength disclinations (Fig. 1a). By observing the half-strength disclinations, it is clear that the surface tilt angle is close to zero; in other words the director lies in the plane of the substrate. As the sample temperature is increased, at a precise temperature which depends on the conditions under which the sample was prepared, a homogenous-to-tilted anchoring transition occurs. This transition is marked by several changes in the sample. First, the apparent birefringence decreases rapidly,

indicating that the optical axis tilts towards the propagation direction. Second, domain boundaries suddenly appear because of the reduced symmetry of the system (from C_2 to C_1). The director is tilted by $-\theta$ on one side of the boundary and $+\theta$ on the other. Although the boundary may form a closed loop, it can also terminate at half-strength disclinations⁸ (Fig. 1b). With further temperature increase, the magnitude of the tilt angle continues to change until a second transition is observed, at which the surface symmetry changes from C_1 to C_∞ and the sample becomes homeotropic ($\theta=90^\circ$). This is shown in Fig. 1c, which is essentially black because the optical axis lies along the propagation direction. On further heating, at a temperature very close to the nematic-to-isotropic transition temperature, we observe another transition associated with surface wetting by the isotropic phase. The onset of this transition is indicated by the reappearance of light transmission (Fig. 1d), as the director is no longer normal to the surface in the isotropic phase. This phase transition may be similar to that observed by Yokoyama *et al.*⁹. The small value of the birefringence suggests that a very thin region of the nematic phase remains in a plane parallel to the surface, roughly in the middle of the cell, surrounded by isotropic liquid. The nematic layer is then analogous to a freely suspended liquid-crystal film. The thickness of the region decreases with increasing temperature, but it does not go continuously to zero. Just below the bulk melting temperature, the nematic layer develops holes and the two isotropic regions on either side merge.

We have measured the surface tilt angle using oriented samples in the temperature region where the liquid crystal undergoes an anchoring transition. We obtain a mono-domain sample by gentle uniform rubbing in a chosen direction. The cells were assembled by placing the two surfaces such that the rubbing surfaces were antiparallel to each other. In this configuration the tilt angle can be measured, if the temperature dependence of the two refractive indices are known, by measuring the effective birefringence and relating it to the transmitted light between cross-polarizers. In this case the transmitted light intensity $I(T)$ with the optic axis at 45° with respect to the polarizer at temperature T is given by $I(T) = I_0 \times \sin^2 [2\pi(n_{\text{eff}}(T) - n_0(T))/d \times \lambda]$, where d is the sample thickness, $n_0(T)$ is the ordinary index, $n_{\text{eff}}(T)$ is the effective extraordinary index, and λ is the wavelength of light. This value of n_{eff} can be used to calculate the tilt angle Θ using the relationship $\Theta = \cos^{-1} [n_0^2 / (n_e^2 - n_0^2) (n_e^2 / n_{\text{eff}}^2 - 1)]$, assuming that the optic axis is uniformly tilted throughout the sample. Figure 2 shows the measured transmitted light intensity as the temperature is changed. It was verified by temperature cycling that the data were very reproducible and that there was no temperature hysteresis. The two symbols represent the data during

FIG. 3 Tilt angle as a function of temperature as determined from the data in Fig. 2. The insert is a semi-log plot of tilt angle against the square root of the reduced temperature.



the cooling and the heating cycle. Figure 3 shows the tilt angle as a function of temperature. If the transition from homeotropic to tilted orientation is second order, then according to Landau theory the tilt angle should scale as $(T_c - T)^{1/2}$. This argument is based on using the tilt angle Θ as the order parameter and expanding the free energy in terms of its even powers¹⁰. The insert in Fig. 3 suggests that this is the case if one chooses T_c to correspond to the onset of the homeotropic transition temperature.

Several other liquid crystals show this behaviour. Although most of the detailed studies reported here have been done with the polar liquid crystal E7, we have also observed similar behaviour with M24 (Merck), S3 MBBA and a nematic liquid crystal composed of centre-symmetric molecules. The mechanism of this anchoring transition is not yet clear, but we suspect that two competing temperature-dependent forces are involved, one that prefers the homogeneous orientation and the other that prefers homeotropic. □

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Abrupt increase in Greenland snow accumulation at the end of the Younger Dryas event

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THE warming at the end of the last glaciation was characterized by a series of abrupt returns to glacial climate, the best-known of which is the Younger Dryas event¹. Despite much study of the causes of this event and the mechanisms by which it ended, many questions remain unresolved¹. Oxygen isotope data from Greenland ice cores²⁻⁴ suggest that the Younger Dryas ended abruptly, over a period of about 50 years; dust concentrations^{2,4} in these cores show an even more rapid transition (≤ 20 years). This extremely short timescale places severe constraints on the mechanisms underlying the transition. But dust concentrations can reflect

subtle changes in atmospheric circulation, which need not be associated with a large change in climate. Here we present results from a new Greenland ice core (GISP2) showing that snow accumulation doubled rapidly from the Younger Dryas event to the subsequent Preboreal interval, possibly in one to three years. We also find that the accumulation-rate change from the Oldest Dryas to the Bølling/Allerød warm period was large and abrupt. The extreme rapidity of these changes in a variable that directly represents regional climate implies that the events at the end of the last glaciation may have been responses to some kind of threshold or trigger in the North Atlantic climate system.

We report accumulation-rate data from the Greenland Ice Sheet Project II (GISP2) ice core, which is being drilled at 72.6° N, 38.5° W, at an elevation of 3,200 m. The core has reached a depth of 2,250 m, about 75% of the ice-sheet thickness. The parallel Greenland Icecore Project (GRIP) core was completed close to bedrock in the summer of 1992 (ref. 3), about 30 km east of the GISP2 drill site. Multi-parameter analyses of both of these cores are yielding high-resolution records of North Atlantic climate changes^{3,4}.

We reconstruct snow accumulation by identifying annual layers in the ice core. Primary indicators (Table 1) include visible strata produced by summer insolation^{5,6}, direct-current electrical conductivity controlled by annually varying strong acids and dust^{7,8}, laser-light scattering from dust⁹, and isotopic composition responding to temperature^{2,3,8}. These indicators are compared with annually varying chemistry over selected intervals of a few metres (ref. 8; dating and Holocene accumulation will be discussed elsewhere (D. Meese *et al.*, manuscript in preparation)).

The accuracy of counting annual layers is sometimes operator-dependent. In common with all other layer-counting techniques, we lack a completely objective, unequivocal way to check our accuracy for the entire record. We use three approaches to estimate our accuracy (Table 1): internal consistency, comparison to volcanic records, and comparison to other published dates for the end of the Younger Dryas (YD). We find good consistency among annual indicators⁸. Cumulative differences over centuries are typically 1% or less, although there are locally larger percentage differences over shorter times, suggesting randomly distributed errors. Use of electrical conductivity measurements (ECM) and dust data to adjust a visible-only timescale required a cumulative change of <1% over 1,800 years between 335 m and 679 m depth.

We find that visible-only or multi-parameter dating matches various volcanoes dated historically, dendrochronologically or by counting of layers in other ice cores to within 1% or better

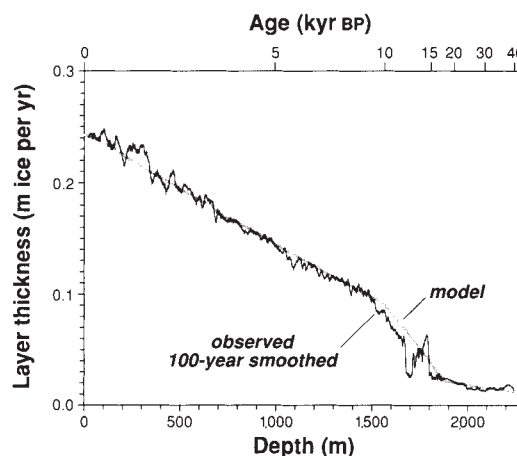
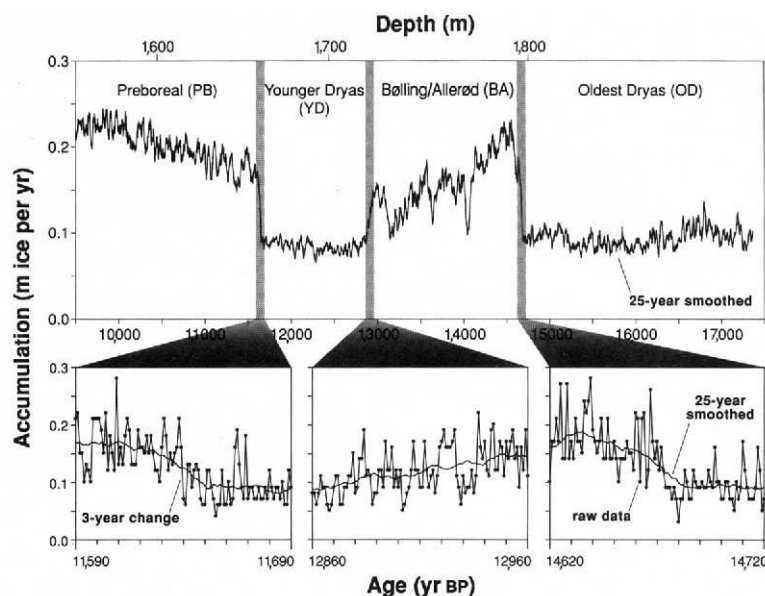


FIG. 1 Measured annual layer thickness in the GISP2 core smoothed with a 100-year running mean, and thicknesses predicted by the model of Schøtt *et al.*^{4,6}.

Fig. 2 Late-glacial accumulation-rate history at GISP2, estimated from data and model in Fig. 1. Ages are in years before 1950. The 25-yr running mean is shown in all panels. Yearly data are shown for the rapid changes at the onsets of the Preboreal (b) and the Bølling/Allerød (d), and the slower change at the onset of the Younger Dryas (c). The arrow in b indicates the possible one- to three-year termination of the Younger Dryas. The resolution in b, c and d is controlled by the 5-mm precision used in recording the layer-thickness data.



over century-length timescales, for the most recent 2,000 years. We used ECM and sulphate to identify volcanic fallout (compare with ref. 7). Some iteration is required; one must know the approximate age of an ECM peak to match it to a historically dated volcano. Several of our peaks over the last 700 years have been verified by comparing major-element chemistry of volcanic glass recovered from the ice core to glass from the suspected volcano (see also refs 10, 11).

We identify the sharp change in layer thickness at 1,678 m depth, corresponding to $11,640 \pm 250$ yr BP (years before 1950) in Figs 1 and 2 with the end of the YD: this depth also is marked by rapid changes in stable-isotopic composition and in dust concentration⁴, with the YD having 'colder' isotopes, more dust and thinner layers than in the younger Preboreal (PB). The YD termination in Europe is dated $\sim 10,000$ – $10,200$ ^{14}C yr BP (refs 12, 13), which has been calibrated to within about two centuries of 11,500 calendar years BP (Table 2). The duration of the YD ($1,300 \pm 70$ years between the midpoints of the onset and termination in the accumulation-rate record) and the timing of the transition from the Oldest Dryas (OD) to the Bølling/Allerød (BA) at $14,680 \pm 400$ yr BP in our record also match U–Th data from corals^{14,15} and new data from the GRIP ice core³ within likely error limits. We believe that these calibration checks confirm our ability to recognize annual layers in the GISP2 core.

Measured annual-layer thickness, λ , allows us to calculate accumulation rate, $\dot{b}$, by correcting for ice flow. Layer thicknesses

and a depth–age scale were modelled¹⁶ for the GISP2 core using an assumed climate history consistent with earlier ice-core data ($\dot{b}_{\text{model}} = 1/3$ of the modern value in the ice age, increasing linearly from 15 to 10 kyr BP to constant modern value of 24 cm ice per year). Modelled layer thicknesses, λ_{model} , are close to observed values except for rapid steps such as the YD (Fig. 1). This allows a first approximation from $\dot{b} = (\lambda/\lambda_{\text{model}})\dot{b}_{\text{model}}$ for each depth (Fig. 2).

The steps in layer thickness were not caused by ice flow. Examination of the core showed no evidence of thrust-faulting or other discontinuities. If anomalous gradients in layer thickness had been caused by enhanced shear strain in soft YD or OD ice¹⁷, there should be rapid or step changes at the climatic termination in the tightness of ice *c*-axis fabrics about the vertical, because that tightness increases with cumulative strain in cold inland ice sheets¹⁸. We measured this tightness by optical and sonic techniques, and found no significant steps in cumulative strain across these boundaries, although the rate of increase of tightness with depth may increase slightly at the YD/PB boundary.

Ice accumulation is low in cold periods (YD and OD) and high in warm periods (PB and BA). The accumulation decrease from BA to YD (Fig. 2c) was gradual, but the increases from YD to PB (Fig. 2b) and OD to BA (Fig. 2d) were abrupt; Broecker¹⁹ has proposed an explanation for this shape in terms of gradual slowdown but abrupt resumption of North Atlantic

TABLE 1 Data used in dating

Depth (m)	Age (yr BP)	Data used	Estimated age error in interval	Estimated age error at bottom (yr)
0–719	0–3,300	visible + ECM, +isotopes 0–300, +laser dust 372–719m; limited chemistry	2%	± 70
719–1,371	3,300–8,020	visible, occas. intercompar. with ECM + laser dust	2%	± 160
1,371–1,510	8,020–9,380	visible + ECM + laser dust	2%	± 190
1,510–1,678	9,380–11,640	visible, occas. intercompar. with ECM + laser dust	2%	± 250
1,678–1,867	11,640–17,380	visible, occas. intercompar. with ECM + laser dust	5%	± 520
1,867–2,250	17,380–40,500	visible occas. intercompar. with ECM + laser dust; 1/10 or 1/20 of metres counted	10%?	$\pm 3,000?$

Recovery of intact core exceeded 97%, and visible logging of this intact core was essentially continuous. 'Occasional intercomparison' refers to checks of about 1 m in 50, usually made in the field. Estimated counting uncertainty on the century to millennial scale (fourth column) is believed to be smallest in Holocene ice where physical properties control the visible signal, acids control the ECM signal and dust controls the laser signal, larger in deeper ice where all three methods respond primarily to dust, and larger still where only 1 m in 10 or 1 m in 20 was counted in the field and interpolations were used; planned counting of the metres skipped in this deepest ice should reduce the uncertainties significantly. We have tried to be slightly conservative in our error estimates.

TABLE 2 Recent dates for end of Younger Dryas

YD end (yr BP)	Method	Ref.	Comment
11,640 ± 250*	GISP2 ice core, Greenland layer count	this work	also Meese <i>et al.</i> manuscript in preparation
11,550 ± 70*	GRIP ice core, Greenland layer count	3	isotopic, other data; preliminary
11,700*	U-Th calibration of ^{14}C	14	
11,200–11,500*	U-Th calibration of ^{14}C	15	
11,000–11,700*	Lake Gosciarz varve calibration of ^{14}C	23	also sharp change in oxygen isotopes
>11,300†	tree-ring calibration of ^{14}C	24, 25	
>10,720 ± 150†	Dye 3 ice core, Greenland	22	our interpretation of published data
10,500–10,800‡	Swedish varve calibration of ^{14}C	26	preliminary
10,630‡	Lake Holzmaar varve date for pollen changes	27	any error from misidentification of turbidites or indistinct layers in some regions not quantified
11,500 ± 200	Average of entries marked with asterisk(*); agrees with †, not ‡	this work	likely age of YD termination

The European radiocarbon age of 10,000–10,200 ^{14}C yr BP^{12,13} has been calibrated by U-Th, tree rings and lake varves. The ice-core records and some of the lake-varve records directly date climate events associated with the YD termination. We treat U-Th years, tree-ring years, varve years and ice-core years as equivalent to calendar years, although recognizing that errors are different for different techniques. The 10,720 yr BP date for the YD/PB transition from the Dye 3, Greenland ice core²² is an undercount, as shown by high-resolution laser-dust calibration of the ECM data used (Fig. 2 of ref 22), probably because current-spreading between the ECM electrodes reduced the resolution below the thickness of some annual layers in the deeper part of the Dye 3 core dated by ECM; our ECM^{4,8} experiences a similar problem for slightly thinner layers, but these occur at more than ~2,000 m depth and 25,000 yr BP at GISP2.

Deep Water formation. Doubling of accumulation from YD to PB seems to have followed a precursor event and to have occurred in three years with most of the change in one year (arrow in Fig. 2b); accumulation increased more than twofold from OD to BA almost as rapidly. From YD to PB, the 25-year running mean increased by 80% in 25 years and doubled in 41 years; thus, even a conservative interpretation shows a transition in accumulation of similar rapidity to that in dust at Dye 3 (ref. 2), and an oscillatory, faster transition similar to ones in the GISP2 ECM record⁴ seems more likely.

Average accumulation on an ice sheet should vary with the temperature-derivative of the saturation vapour pressure at cloud level if dynamical processes in the atmosphere do not change²⁰. If so, the twofold YD/PB accumulation increase requires a warming of ~7 °C for assumed PB precipitation temperature equal to the modern mean annual surface temperature of -31 °C; this estimate would change only ±1 °C for ±17 °C change in assumed precipitation temperature.

Dansgaard *et al.*² report an isotopic change in the Dye 3 core from YD to PB equivalent to 7 °C warming; similar or slightly smaller isotopic changes occur in the GRIP³ and GISP2 cores⁴. Dansgaard *et al.*² also interpret a rapid change in deuterium excess from YD to PB as indicating a significant cooling of the oceanic vapour source, as sea-ice retreat exposed a large region of cold ocean water. Isotopic composition of snow depends among other factors on the temperature difference between the

vapour source and the site of snow formation²¹, so some of the isotopic change in Greenland ice may reflect this oceanic cooling, leaving 7 °C as an upper limit for ice-sheet warming from YD to PB. But because the full 7 °C warming is required to explain the observed accumulation-rate change thermodynamically, it is likely that accumulation was affected by changes in atmospheric dynamics, such as an increase in the number or duration of precipitating weather systems from YD to PB. Such an increase might have occurred as the storm track followed the sea-ice edge northward towards Greenland from YD to PB².

It has been proposed that late-glacial climate oscillations including the YD are linked to the thermohaline circulation of the oceans through changes in deep-water formation in the north Atlantic and associated changes in advection of warm surface water (see for example ref. 19). The rapidity of the YD/PB and OD/BA transitions in accumulation rate, an important climate variable, together with the possibly oscillatory nature of the accumulation-rate changes and the clearly oscillatory nature of the ECM and dust record⁴, place severe constraints on models of these events. In particular, such climate variables as whole-ocean salt budgets, insolation, atmospheric CO₂ concentration and ice-sheet size are unlikely to have changed as rapidly as this. Causes must be sought in threshold levels in the climate system or in especially fast-acting components of the climate system including restricted, sensitive 'trigger' regions of deep oceanic convection, and atmospheric circulation patterns. □

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Separating mantle from slab signatures in arc lavas using B/Be and radiogenic isotope systematics

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At convergent margins, tectonic processes juxtapose subducted slab, mantle wedge and the crust of the upper plate in a column beneath the overlying arc volcano. As each of these components is expected to be chemically heterogeneous, and as all may contribute to magma chemistry, identifying the different sources of arc magmas has been difficult. A working hypothesis has emerged, in which tholeiitic and calc-alkaline lavas in island arcs are partial melts of the mantle produced by fluxing of the wedge by hydrous fluids from the subducted slab^{1,2}. Trace-element and radiogenic isotope ratios have been used to define the chemical characteristics of these sources but cannot be unequivocally identified with one source; by contrast, high B/Be and ¹⁰Be/⁹Be ratios in arc lavas uniquely identify the subduction component^{3,4}, and thus separate chemical variability owing to recent subduction from that reflecting other causes. Here we combine B/Be with Sr, Nd and Pb isotope systematics of alkaline, calc-alkaline and tholeiitic lavas from Java and Flores, Indonesia, to constrain the isotopic composition of their mantle and subduction sources. The alkaline lavas always have low B/Be, from which we conclude that they are derived from mantle that has not been modified by recent subduction (in agreement with refs 5 and 6). We also show that the isotopic composition of the subducted component is relatively homogeneous along the length of the arc, suggesting that the subduction of Australian continental lithosphere in the east started too recently to have changed the nature of the subducted material at present beneath Flores.

The volcanoes studied here span 1,500 km of arc length and two very different tectonic settings. In Java, oceanic lithosphere of the Indian plate is subducting beneath Eurasia at a rate⁷ of 6 cm yr⁻¹ (Fig. 1). Flores is situated in an arc-continent collision zone and the Australian continental lithosphere is subducting beneath the arc at a rate⁷ of 3 cm yr⁻¹; the depth to which continental crust persists is not known. Upper-plate characteristics also change along the arc, from quasi-continental crust at Java to oceanic at Flores (Fig. 1). The tholeiitic and calc-alkaline lavas in Java and Flores have been erupted from volcanoes situated 118–192 km and 90–120 km, respectively, above the Benioff zone⁷. The alkaline lavas discussed here are intimately associated with the calc-alkaline lavas and are erupted from volcanoes situated just behind this main volcanic front, at 185–210 km above the Benioff zone⁷.

The volcanic rocks of the Sunda arc vary widely in K₂O content, and include tholeiitic, calc-alkaline, high K calc-alkaline and potassic alkaline (leucititic and shoshonitic) members^{5,8}. The tholeiitic and calc-alkaline lavas from Java and Flores have many chemical characteristics typical of arc lavas, including high concentrations of Al₂O₃ (>13 wt%), low levels of TiO₂ (<1.2 wt%) (ref. 9), large ion lithophile (LILE) and light rare earth element (LREE) enrichment relative to heavy rare earth (HREE) and high field strength elements (HFSE), negative Nb anomalies, low Ce/Pb ratios (2–10) and high Ba/La ratios (>13) (refs 5, 6, 10, 11). The alkaline lavas have low concentrations of TiO₂, similar to arc lavas, but have lower LILE/ and LREE/ HFSE ratios, small to negligible Nb anomalies, higher Ce/Pb ratios (5–35) and a range of Ba/La ratios (3–72) (refs 6, 10–13).

Many of these 'arc characteristics' are thought to reflect, sometimes indirectly, the role of subduction in arc magmatism. Elevated B/Be ratios (10–170) are observed in almost all island arc tholeiitic and calc-alkaline lavas^{4,14–16}; positive correlations with the ¹⁰Be/⁹Be ratio (ref. 4) indicate that the B/Be ratio may be used as a direct indicator of contributions from the subducted plate. Ocean sediments and the altered fraction of oceanic crust have high B/Be ratios (~100 and 30–170 respectively), whereas mid-ocean-ridge basalts (MORB) and ocean island basalts (OIB), even those with high μ (HIMU) and enriched mantle II (EMII)¹⁷ affinities, have low and indistinguishable B/Be ratios of 3–5 (refs 14, 16). B/Be ratios in MORB and OIB are always low, irrespective of the concentration of

FIG. 1 Tectonic map of the Sunda arc after Hamilton (1979)⁷. The quasi-continental crust is oceanic in composition and continental in thickness. Note the changes in thickness and composition of the upper plate, and the duration of subduction, along the strike of the arc. G: Guntur; C: Cereme; RB: Ringgit-Beser; KM: Keli-Mutu; L: Lewotobi; M: Mandiri; W: Watukuba.

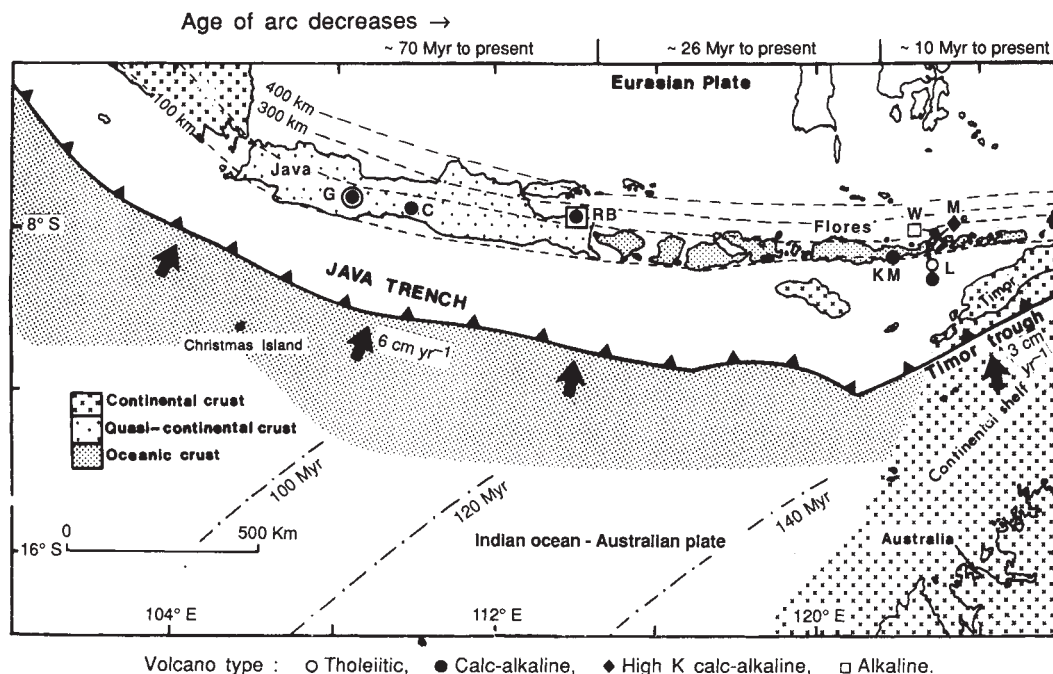


TABLE 1 Composition of lavas

Sample	Comp.	SO ₂ wt. %	K ₂ O, wt. %	B, p.p.m.	Be, p.p.m.	B/Be	¹⁰ Be × 10 ⁶ atoms g ⁻¹	⁸⁷ Sr/ ⁸⁶ Sr	¹⁴³ Nd/ ¹⁴⁴ Nd	²⁰⁶ Pb/ ²⁰⁴ Pb	²⁰⁷ Pb/ ²⁰⁴ Pb	²⁰⁸ Pb/ ²⁰⁴ Pb
Guntur												
Gu1	Tholeiite	49.97	0.33	5.0	0.53	9		0.70393	0.512961	18.622	15.640	38.87
Gu5	"	51.63	0.51	5.7	0.49	12	0.3	0.70404	0.512896	18.663	15.644	38.91
Gu7	"	51.45	0.55	5.7	0.51	11		0.70430	0.512887*	18.690*	15.654	38.98
Gu9	"	51.20	0.52	5.6	0.52	11		0.70408	0.512902*	18.688*	15.657	38.98
Gu2	Calc-alk	61.16	1.85	31.2	0.61	51		0.70472	0.512728	18.833	15.691	39.21
Gu3	"	63.38	2.15	37.1	0.75	49		0.70509	0.512697	18.853	15.704	39.27
Gu10	"	58.35	1.48	19.1	0.57	34	0.3	0.70441	0.512781	18.811	15.686	39.18
Cereme												
Ca7	Calc-alk	50.71	1.07	6.2	0.44	14		0.70475	0.512708	18.726	15.666	39.02
Ca15	"	57.17	1.28	6.0	0.82	7	0.3	0.70447	0.512752			
Ca22	"	54.78	1.48	10.3	0.53	19	0.2	0.70476		18.725	15.673	39.03
Ca40	"	51.58	1.00	4.1	0.67	6		0.70462	0.512731			
Ringgit												
R57	Calc-alk	47.85	1.17	12.0	0.50	24		0.70422	0.512821	18.667	15.631	38.97
R59	"	46.88	0.98	5.4	0.43	13		0.70418	0.512842	18.720	15.647	39.03
R30	HK CA	53.94	2.92	12.7	1.73	7		0.70462	0.512759*	18.754*	15.672	39.15
R64	"	48.07	2.15	10.6	0.92	12		0.70463	0.512729	18.596	15.624	39.02
R18	Potassic	47.35	5.92	27.5	5.78	5		0.70496	0.512674	18.739	15.623	39.03
R28	series	51.20	5.56	43.3	10.7	4		0.70457	0.512731*	18.712	15.641	39.12
R36	"	45.06	4.14	7.8	9.64	1		0.70449	0.512779	18.915	15.627	39.10
R43	"	53.49	4.19	12.0	6.07	2		0.70463	0.512754	18.719	15.624	39.05
R45	"	45.50	2.79	9.2	1.87	5		0.70469	0.512806	18.631	15.622	39.04
R47	"	52.83	6.15	33.5	6.88	5		0.70445	0.512696*	18.611	15.620	39.01
R5	Enriched	46.16	5.27	1.9	1.43	1		0.70478	0.512684	18.546	15.627	39.18
R15	potassic	43.90	3.88	7.6	1.63	5		0.70458	0.512692	18.513	15.617	39.14
R27	series	44.86	5.76	3.6	2.54	1		0.70468		18.493	15.626	39.19
R38	"	46.58	5.09	7.9	1.72	5		0.70478	0.512726*	18.498	15.633	39.18
R39	"	42.86	4.72	4.7	1.35	3		0.70463	0.512741*	18.570*	15.628	39.15
Lewotobi												
L29	Tholeiite	56.22	0.80	11.9	0.52	23		0.70527	0.512817	18.970*	15.670	39.34
L33	"	60.37	0.96	14.9	0.52	29	0.4	0.70517	0.512834	18.960*	15.670	39.29
L35	"	52.91	0.56	15.0	0.66	23		0.70548	0.512767	18.990*	15.690	39.39
L36	"	57.54	0.81	17.0	0.65	26		0.70524				
L37	"	54.68	0.94	14.3	0.59	24		0.70543				
L1	Calc-alk	58.73	1.37	16.4	0.65	25		0.70580	0.512763*	19.012*	15.681	39.42
L5	"	57.11	1.26	14.0	0.68	21		0.70599				
L10	"	59.90	1.42	15.7	0.69	23	0.1	0.70584	0.512760	19.000*	15.680	39.41
L12	"	55.55	1.19	15.1	0.59	26		0.70605	0.512736*	19.026*	15.687	39.44
L19	"	59.06	1.48	15.3	0.63	24		0.70566				
L25	"	55.67	1.19	13.2	0.59	22		0.70563	0.512764	19.009*	15.674	39.38
L28	"	55.71	1.13	10.5	0.73	14		0.70618	0.512734	19.010*	15.690	39.42
Keli Mutu												
K7	Calc-alk	59.81	1.70	69.4	0.67	104		0.70537	0.512796*	18.897*	15.661	39.15
K22	"	61.39	1.75	82.5	0.76	109	0.2	0.70528		18.879*	15.653	39.15
K26	"	54.91	1.01	44.5	0.60	74		0.70548		18.914*	15.689	39.26
K30	"	51.88	0.86	20.4	0.59	35	0.2	0.70530	0.512739	18.917*	15.670	39.21
K33	"	63.44	1.56	35.0	0.79	44		0.70499	0.512819*	18.869*	15.662	39.15
Mandiri												
M4	HK CA	58.98	2.87	10.1	1.37	7		0.70599	0.512707*	18.989*	15.700	39.42
M7	"	61.04	3.74	16.2	1.76	9		0.70651	0.512708*	18.970*	15.676	39.38
M11	"	50.01	1.56	7.4	1.17	6		0.70638	0.512671*	18.982*	15.682	39.39
M19	"	48.25	1.14	4.9	0.79	6		0.70623	0.512649	18.970	15.682	39.39
M20	"	56.10	2.50	10.5	1.44	7		0.70656	0.512659	18.950*	15.664	39.34
Watukubu												
W7	Shosh.	47.80	2.08	4.3	2.45	2		0.70508	0.512696*	18.838*	15.618	39.18
W8	"	53.97	2.56	4.7	1.49	3		0.70539	0.512712*	18.913*	15.641	39.29
W10	"	48.17	1.63	3.2	1.13	3		0.70529	0.512703*	18.957*	15.651	39.35
W14	"	64.07	4.41	15.6	2.10	7		0.70515	0.512701*	18.917*	15.645	39.29
W26	"	50.20	2.32	4.9	1.67	3		0.70455	0.512710	18.852	15.602	39.17

B and Be were determined by standard addition on the inductively coupled plasma atomic emission spectrometer at the Department of Terrestrial Magnetism (DTM), Carnegie Institution of Washington using the techniques described in refs 11, 16. ¹⁰Be was measured by accelerator mass spectrometry at the Tandem Accelerator Lab at the University of Pennsylvania³. Analytical precision for B is ±5% above 10 p.p.m. but drops to ±10% at 1 p.p.m.; for ⁹Be the uncertainty is ±5% at 0.2 p.p.m. Analytical uncertainties for low-concentration ¹⁰Be measurements are typically ±15%. See refs 26, 27 for the techniques of Sr, Nd and Pb isotope analyses. Samples marked with an asterisk were analysed for Nd and/or Pb at DTM. For the samples analysed at DTM ¹⁴³Nd/¹⁴⁴Nd is normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.7219 and the mean for 148 analyses of La Jolla is 0.511842 ± 63; Pb data are corrected for mass fractionation, ratio by ratio, for the difference between the average measured values for the NBS 981 standard and those reported in ref. 28. Uncertainties on Pb isotopic composition are <0.05% per a.m.u. and Pb blanks were <400 pg. For samples analysed at Royal Holloway College (RHC) the ⁸⁷Sr/⁸⁶Sr is normalized to ⁸⁶Sr/⁸⁸Sr = 0.1194, and the standard SRM 987 gave ⁸⁷Sr/⁸⁶Sr = 0.710242 ± 20 (2σ) on 73 analyses for the period of data collection; ¹⁴³Nd/¹⁴⁴Nd is normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.7219 and adjusted to ¹⁴²Nd/¹⁴⁴Nd = 1.141870 (ref. 29). The RHC Nd laboratory standard analysed over the period of data collection gave ¹⁴³Nd/¹⁴⁴Nd = 0.511419 ± 11 (2σ) on 45 analyses, whilst La Jolla gave 0.511857 ± 7 (2σ) on 17 analyses. Pb isotope ratios are normalized for mass fractionation to SRM 981 by ~0.11% per a.m.u. Replicate analyses of SRM 981 give 2 s.e. of ±0.01 for ²⁰⁶Pb/²⁰⁴Pb, ±0.015–0.02 for ²⁰⁷Pb/²⁰⁴Pb and ±0.04 for ²⁰⁸Pb/²⁰⁴Pb. Blanks for the period of analysis were ~1 ng Sr, 0.3 ng Nd and <1.0 ng Pb. These are not significant to the isotopic results.

boron (<3 p.p.m. and <20 p.p.m. respectively). Consequently B/Be ratios in arc lavas are insensitive to the pre-existing composition of the mantle wedge, and are strongly influenced by the subduction zone component (sediment + altered oceanic basalt (refs 4, 14, 16)). Beryllium and boron have similar bulk distribution coefficients in arc lavas¹⁵, and the B/Be ratio is largely unaffected by partial melting and fractional crystallization. Enhanced solubility of B relative to Be in hydrous fluids is expected however, and this could change ratios^{1,18,19}, consistent with the observed decreases in B and B/Be in progressively metamorphosed subducted sediment and altered ocean basalt^{19,20}. The high B/Be ratios in arc lavas implies that preferential extraction of B from the slab has taken place, probably by a hydrous fluid. The residence time of B in the sub-arc mantle

is estimated⁴ to be ≤ 5 Myr. If this is correct only those lavas derived from mantle that has been recently modified by subduction will have high B/Be ratios; mantle modified by older subduction will produce lavas with B/Be ratios similar to MORB and OIB. Only in the Cascades do calc-alkaline lavas have low B/Be ratios (<5); this is attributed to the subduction of a young, hot plate, where dehydration and B transfer out of the slab are thought to occur at levels significantly shallower than those of magma generation²¹.

The suite of lavas studied here show the influence of both the heterogeneous mantle and the subducted slab. The B/Be ratios of tholeiitic and calc-alkaline lavas from Java and Flores range from 8–109, but are always <7 for the alkaline lavas, as shown in Table 1. (Muriah is not included in this sample set, because

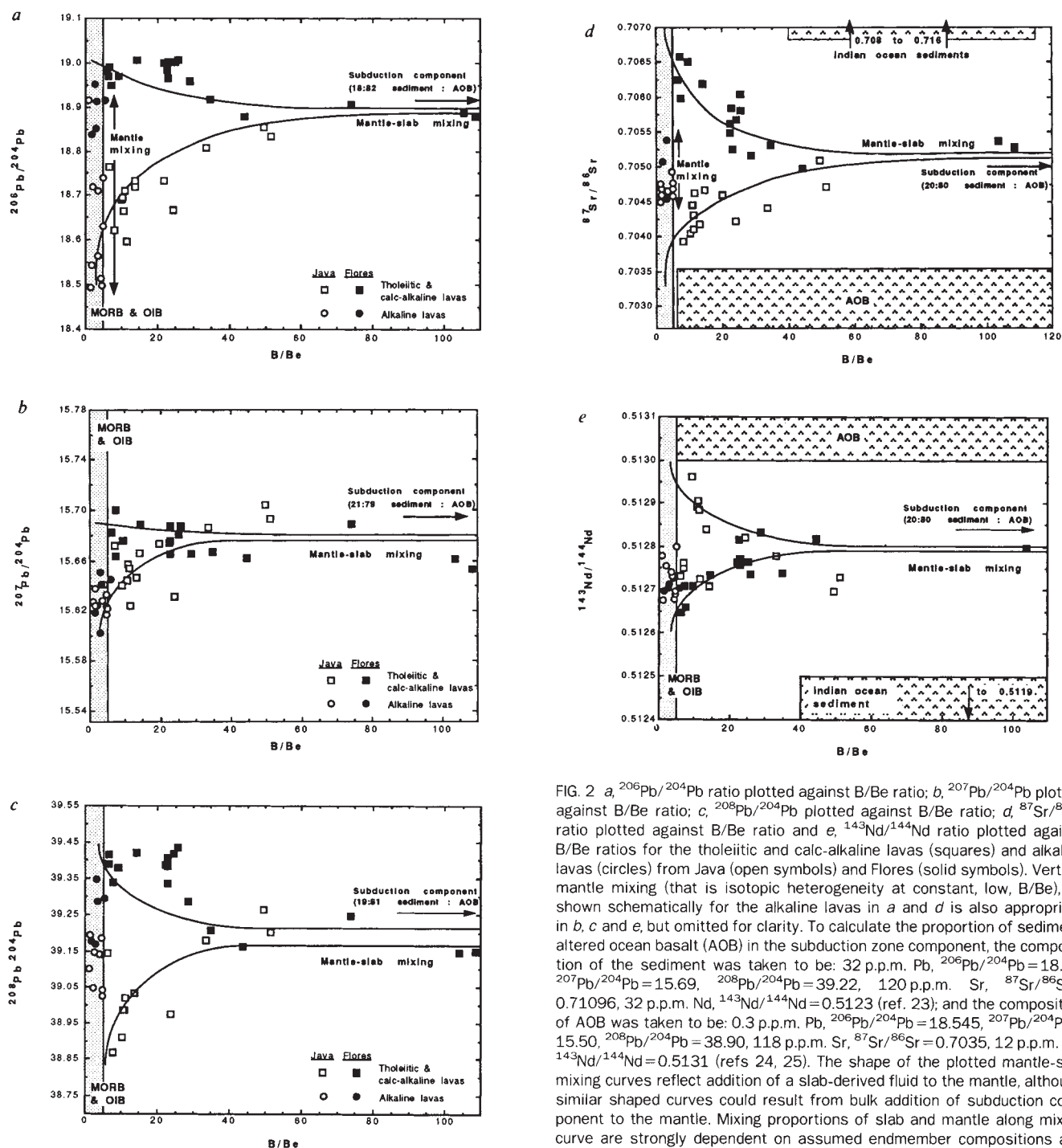


FIG. 2 a, $^{206}\text{Pb}/^{204}\text{Pb}$ ratio plotted against B/Be ratio; b, $^{207}\text{Pb}/^{204}\text{Pb}$ plotted against B/Be ratio; c, $^{208}\text{Pb}/^{204}\text{Pb}$ plotted against B/Be ratio; d, $^{87}\text{Sr}/^{86}\text{Sr}$ ratio plotted against B/Be ratio and e, $^{143}\text{Nd}/^{144}\text{Nd}$ ratio plotted against B/Be ratios for the tholeiitic and calc-alkaline lavas (squares) and alkaline lavas (circles) from Java (open symbols) and Flores (solid symbols). Vertical mantle mixing (that is isotopic heterogeneity at constant, low, B/Be), as shown schematically for the alkaline lavas in a and d is also appropriate in b, c and e, but omitted for clarity. To calculate the proportion of sediment: altered ocean basalt (AOB) in the subduction zone component, the composition of the sediment was taken to be: 32 p.p.m. Pb, $^{206}\text{Pb}/^{204}\text{Pb}=18.92$, $^{207}\text{Pb}/^{204}\text{Pb}=15.69$, $^{208}\text{Pb}/^{204}\text{Pb}=39.22$, 120 p.p.m. Sr, $^{87}\text{Sr}/^{86}\text{Sr}=0.71096$, 32 p.p.m. Nd, $^{143}\text{Nd}/^{144}\text{Nd}=0.5123$ (ref. 23); and the composition of AOB was taken to be: 0.3 p.p.m. Pb, $^{206}\text{Pb}/^{204}\text{Pb}=18.545$, $^{207}\text{Pb}/^{204}\text{Pb}=15.50$, $^{208}\text{Pb}/^{204}\text{Pb}=38.90$, 118 p.p.m. Sr, $^{87}\text{Sr}/^{86}\text{Sr}=0.7035$, 12 p.p.m. Nd, $^{143}\text{Nd}/^{144}\text{Nd}=0.5131$ (refs 24, 25). The shape of the plotted mantle-slab mixing curves reflect addition of a slab-derived fluid to the mantle, although similar shaped curves could result from bulk addition of subduction component to the mantle. Mixing proportions of slab and mantle along mixing curve are strongly dependent on assumed endmember compositions and presumed mixing processes, and so are not shown.

it is located far behind the volcanic front, above a slab some 360 km deep; for comparison, however, the mean of 12 B/Be ratios for Muriah is 6.7 ± 2 .) Comparison of B/Be ratios with $^{206}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$, $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for the Indonesian lavas (Fig. 2) shows that the alkaline lavas span a wide range of $^{206}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios, all at the low B/Be ratios typical of MORB and OIB. Now because Guntur (some 170 km above the slab) has erupted lavas with B/Be as high as 50, and because high B/Be calc-alkaline lavas are interlayered with low B/Be alkaline lavas at Ringgit-Beser (210 km above the slab), the low B/Be ratios of the alkaline lavas in this study cannot reflect loss of B from the slab at shallower levels. We conclude that the alkaline lavas are derived from localized portions of the mantle which has not been affected by recent subduction modification, in keeping with conclusions derived elsewhere from other lines of evidence^{5,6}. Moreover, calculations show that the large isotopic differences between these mantle sources cannot be due to addition or removal of the parent nuclides through enrichment or depletion events over the lifetime of the arc; thus mixing of distinct mantle components, which do not carry a modern subduction signature, is required¹¹.

The tholeiitic and calc-alkaline lavas from Java and Flores define distinct trends which can be represented by two hyperbolic mixing curves that converge at high B/Be (>30 , Fig. 2) indicating the near homogeneity of the subduction component along the length of the arc, and emphasizing the isotopic differences in the mantle endmember sampled in the two regions. Mixing curves are least well defined for $^{207}\text{Pb}/^{204}\text{Pb}$, where the total variation is small relative to the fractionation uncertainty of ± 0.02 on each measurement. Lavas from Java are produced by mixing of a subduction zone component with a mantle source characterized by low $^{206}\text{Pb}/^{204}\text{Pb}$, $^{207}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$, $^{87}\text{Sr}/^{86}\text{Sr}$ and high $^{143}\text{Nd}/^{144}\text{Nd}$ (Fig. 2). The Flores lavas are produced by mixing between a more enriched mantle and a subduction component closely similar to that at Java (Fig. 2). The Flores mixing hyperbola is especially well defined and constrains the subduction component there to have $^{143}\text{Nd}/^{144}\text{Nd} = 0.51273\text{--}0.51282$, $^{87}\text{Sr}/^{86}\text{Sr} = 0.7050\text{--}0.7055$, $^{206}\text{Pb}/^{204}\text{Pb} = 18.85\text{--}18.90$, $^{207}\text{Pb}/^{204}\text{Pb} = 15.66\text{--}15.69$ and $^{208}\text{Pb}/^{204}\text{Pb} = 39.15\text{--}39.25$. These values are distinct from those expected if the subducted component were derived from north-west Australian continental crust which is mid-Proterozoic and older⁷ in age, and imply instead a mixture of sediment and altered basaltic crust derived from a subducted oceanic plate. The Pb, Sr and Nd isotopic characteristics of the subduction component, defined by the mixing curves for both Java and Flores, can be produced by mixing $\sim 20\%$ Indian Ocean sediment and 80% altered oceanic basalt (Fig. 2). Some scatter about the mixing curves (particularly for Nd) allows some hetero-

geneity in the endmember values chosen; for the subduction component, however, the allowed heterogeneity is small relative to the total range of isotopic variation seen in the subducted plate (Fig. 2).

Our ^{10}Be data are all measured on samples (including Keli-Mutu) where multiple leaching shows no leachable ^{10}Be of the sort expected from surface alteration. Like previously published values³, these new data show extremely low concentrations of ^{10}Be (≤ 0.4 million atoms per g) meaning that ^{10}Be data cannot be combined with B data as employed elsewhere⁴ either to constrain the relative proportions of young sediment and altered oceanic basalt in the subduction component, or as a check for the homogeneity of the subduction zone component. Low ^{10}Be concentrations in the Indonesian lavas require only that any subducted sediment involved in magma genesis be too old to contain ^{10}Be .

The approximate homogeneity of the subduction zone component implied here (and elsewhere⁴) is important for the lavas of the Sunda arc, given the change in the subducting plate along the arc. Assuming present convergence rates and arc geometry, sediments currently involved in magma generation were supplied to the trench about 3.5 Myr ago; these sediments may have been roughly the same everywhere. If so, the supply of continental crust to depth, associated with the arc-continent collision, is more recent than 3.5 Myr. This conclusion is in contrast to that reached on the basis of a He isotope study, although the only Flores volcano with both B/Be and He measurements (Keli-Mutu) has $R/\text{Ra} = 6.0$ ($R/\text{Ra} = {}^3\text{He}/{}^4\text{He}$ of the sample divided by ${}^3\text{He}/{}^4\text{He}$ of air) in the range reported for Java²². The large isotopic variability within the sediment column at any site²³ requires the processing of heterogeneous material to give a roughly homogeneous subduction component as sampled by the lavas⁴. It is difficult, however, to see how any mechanical or chemical process could operate over 1,500 km of arc length, leading us to prefer a model in which the arc-continent collision is sufficiently recent to ensure that the region of magma generation at Flores has not yet been affected by deep subduction of continental lithosphere.

The production of alkaline lavas with low B/Be ratios implies that there are one or more mantle components that are fertile, hot or deep enough to melt without significant (and recent) fluxing from the subducted slab. The close spatial and temporal relationship between these alkaline lavas and the interlayered calc-alkaline lavas (with higher B/Be) at Ringgit-Beser suggests that the mantle components from which the alkaline lavas are derived are close to mantle that has been both fluxed and melted. Moreover, mixing of the alkaline and calc-alkaline magmas is limited, as few examples of mixed magmas are erupted, suggesting that the plumbing for the two magma types remains separate. $\square$

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Three new human skulls from the Sima de los Huesos Middle Pleistocene site in Sierra de Atapuerca, Spain

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THREE important fossil hominids were found in July 1992 in the Middle Pleistocene cave site called Sima de los Huesos (Sierra de Atapuerca, Burgos, Northern Spain). One is a complete calvaria (cranium 4), the second a virtually complete cranium (cranium 5), the third represents a more fragmentary cranium of an immature individual (cranium 6). There is a large difference in size between the two adult specimens (for example endocranial volume 1,125 cm³ versus 1,390 cm³). The Atapuerca human remains are dated to >300,000 years. The Atapuerca cranial sample fits within the 'archaic *Homo sapiens*' group, but is well differentiated from the Asian *Homo erectus* group. The extensive Atapuerca human collection is the most complete sample of Middle Pleistocene humans yet discovered from one site, and appears to document an early stage in Neanderthal evolution.

The skulls (Fig. 1) were discovered in a bone-bearing breccia in the Sima de los Huesos cave site (Fig. 2). The Sima de los Huesos human sample so far recovered amounts to more than 700 fossils, belonging to at least 24 individuals (based on dental evidence, refs 1, 2 and J. M. Bermúdez de Castro, personal communication). The human fossils are mixed and without anatomical connections, but all skeletal elements are represented in the sample, without bias³. The absence of herbivore bones or stone implements in the site indicates the human fossils do not derive from a camp site or carnivore activity. The human bone accumulation, therefore could be anthropic or catastrophic³. The faunal composition of the breccia is mainly *Ursus deningeri*⁴ (more than 250 individuals). There are also a few fossils of carnivores: *Panthera leo*, *Panthera cf. gombaszoegensis*, *Lynx pardina spelaea*, *Vulpes vulpes*, Canidae sp., Mustelidae sp.^{5,6}. A speleothem overlying the human fossils has been dated to >300,000 years (300 kyr) (J. Bischoff, personal communication) (Fig. 2).

The cranial capacities of the Atapuerca adult specimens have been measured three times using millet seed, giving a figure of 1,390 cm³ (±10 cm³) for cranium 4 and 1,125 cm³ (±10 cm³) for cranium 5. Thus, although cranium 5 aligns with the smallest European or African Middle Pleistocene specimens (for example Steinheim, Ndutu), the cranial capacity of cranium 4 is one of the largest in the Middle Pleistocene record. On the basis of metrical comparisons with the two adult individuals, the cranial capacity of the immature individual is estimated at around 1,100 cm³. The vault is high when observed in norma lateralis in cranium 4 (height/length index = 65.2) and cranium 5 (height/length index = 67.6; Neanderthal range = 60.0–66.6, *N* = 10; refs 7–10). The maximum cranial breadth of cranium 4 (164 mm) is much greater than in cranium 5 (145 mm) and is among the largest known in the human fossil record.

In the Atapuerca sample the occipital torus is straight in inferior and posterior views, fading towards the asterion. There is always a well defined rough and/or porous surface above the occipital torus, more or less flat but never depressed (which seems to foreshadow the suprainiac fossa found in Swanscombe¹¹ or in Neanderthals). In crania 4, 5 and 6, the opisthocranium lies at the upper margin of this suprainiac surface, but

the cranial length is only slightly less at the nuchal torus. The lambda-inion-opisthion angles of cranium 4 (112.9°) and cranium 5 (116.5°) fall between the values of Petralona and Swanscombe¹¹ (Neanderthal range = 122.5°–129°, *N* = 4; ref. 8). The ratio (lambda-inion arc/lambda-opisthion arc) × 100 is 61.6 in cranium 4 and 56.5 in cranium 5 (Neanderthal range = 54.9–66.9, *N* = 8; refs 7–9).

The mastoid processes are big and projecting in the Atapuerca adult individuals. The juvenile individuals exhibit small mastoids, which project less than the occipitomastoid region, as is common in Neanderthals (both adults and juveniles) and modern human immatures, suggesting retention of the immature condition in Neanderthals.

The morphology of the supraorbital torus is very consistent in the sample: continuity of the lateral, orbital and glabellar segments and inflated glabellar region without a deep midline depression. A similar pattern can be observed in Bilzingsleben, Steinheim and Petralona, but not in Arago 21 (which resembles Broken Hill and Bodo). The supraorbital torus is well developed in all the specimens of the Atapuerca sample¹², including juveniles. Thickness at the midorbital and lateral points of the supraorbital torus¹³ in cranium 5 (14.1 mm, 14.6 mm) is greater than in cranium 4 (12 mm, 11.5 mm) (Neanderthal ranges: 8.7–13.9 mm, 9.5–13.3 mm, *N* = 9). The nasal bones are projecting and the nasal root is at the same level as the glabella, as in Bilzingsleben and Neanderthals (but unlike Petralona and Steinheim). The nasiofrontal angle¹⁴ is low (137°).

The total facial prognathism is remarkable in cranium 5. The prosthion angle of the upper facial triangle¹⁴ (60.5°) is close to Petralona (60.2°; ref. 15) and to the lower limit of the Neanderthal range (59°–72.4°, *N* = 7; refs 7–10). The nasoalveolar clivus is inclined and the sagittal and transversal facial angles at subspinale indicate a marked midfacial projection, a derived trait shared with Neanderthals (zygomaxillary angle¹⁴ = 111.2°; Neanderthal range = 105°–125°, *N* = 8; ref. 7). The ratio interorbital breadth/bifrontal breadth is high in cranium 4 (32.6) and cranium 5 (29.5) (Petralona = 28.7, ref. 15; Neanderthal range = 20.1–29.9, *N* = 13; ref. 7). The orbits are small (left side: breadth = 41.2 mm, height = 33 mm) and mesoconchic. Bimaxillary breadth (118.4 mm), cheek height (37 mm) and nasal aperture breadth (38 mm) are very large in absolute terms and even more in relative terms. The inferior nasal rim morphology

BOX 1 Main traits of the Atapuerca cranial sample

Invariant features. Maximum cranial breadth at the supramastoid crest (*N* = 5); opisthocranium ≠ inion (*N* = 3); occipital squama » nuchal plane (*N* = 4); rough and/or porous suprainiac surface (*N* = 5); occipital torus reduced laterally (*N* = 11); high and rounded temporal squama (*N* = 5); absence of anterior mastoid tubercle (*N* = 9); developed postglenoid process (*N* = 7); tympanic highly angled to petrous (*N* = 6); absence of a fissure separating mastoid from tympanic (*N* = 8); not robust tympanic region (*N* = 6); ossified styloid process (*N* = 8); double arched and fused supraorbital torus (*N* = 5).

Variable features. Parietal wall sides convergent towards the top in cranium 4 and parallel in crania 5 and 6; parietal angular torus marked in cranium 4, weak in cranium 5, and absent in cranium 6 and two more specimens; sphenoid contributes to the medial wall of the glenoid fossa in crania 4, 6 and three more specimens and does not contribute in cranium 5 and two more specimens; big and projecting mastoid processes in adult individuals (*N* = 5), but small mastoids in juvenile individuals (*N* = 3); external occipital crest between superior and inferior nuchal lines present in cranium 4 and absent in crania 5, 6 and two more specimens; canine fossa absent in crania 5 and 6 and present in AT-404; zygomaxillary inferior margin high and broadly curving in cranium 5 and high but straight and more oblique in cranium 6 and AT-404.

Traits preserved in few specimens. Vertex not coincident with bregma in cranium 5; slight parietal keeling in crania 4 and 5; frontal keeling slight in cranium 4 and absent in cranium 5; midfacial projection in cranium 5.

is reminiscent of that of Broken Hill. There are no canine fossa in crania 5 and 6 (nor in Petralona and Arago 21). In contrast, a canine fossa is present in Steinheim and the Atapuerca specimen AT-404 (a large fragment of the left side of the face). In cranium 5, the maxillary pneumatization is extensive. The frontal sinuses in the Atapuerca sample are well developed but do not reach the supraorbital notch nor penetrate the frontal squama. Cranium 5 exhibits a high and broadly arching inferior zygomaxillary margin as in Bodo, Petralona, Broken Hill, Arago

21 or Steinheim¹⁶ but there is no maxillary torus (as is clearly seen in Petralona, Arago 21 or Steinheim). The inferior zygomaxillary margin is also high in cranium 6 and AT-404, but it is straight and more oblique than in cranium 5.

Box 1 presents a summary of the morphology of the Atapuerca cranial sample. All appear to exhibit an occipital morphology that anticipates the Neanderthal pattern. The supraorbital torus morphology is Neanderthal-like. There is a clear midfacial projection and an inflated maxilla in cranium 5. Neanderthal

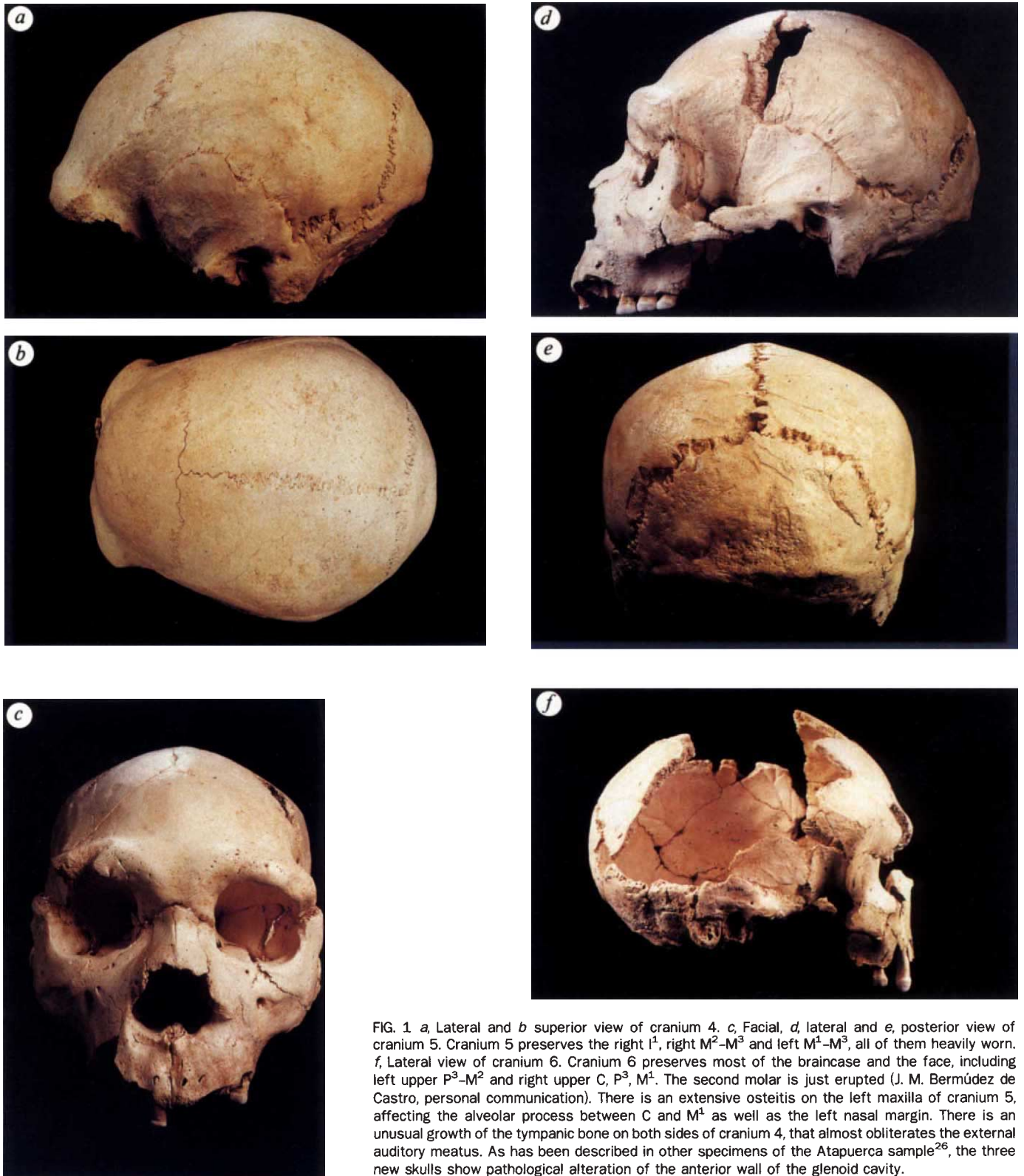
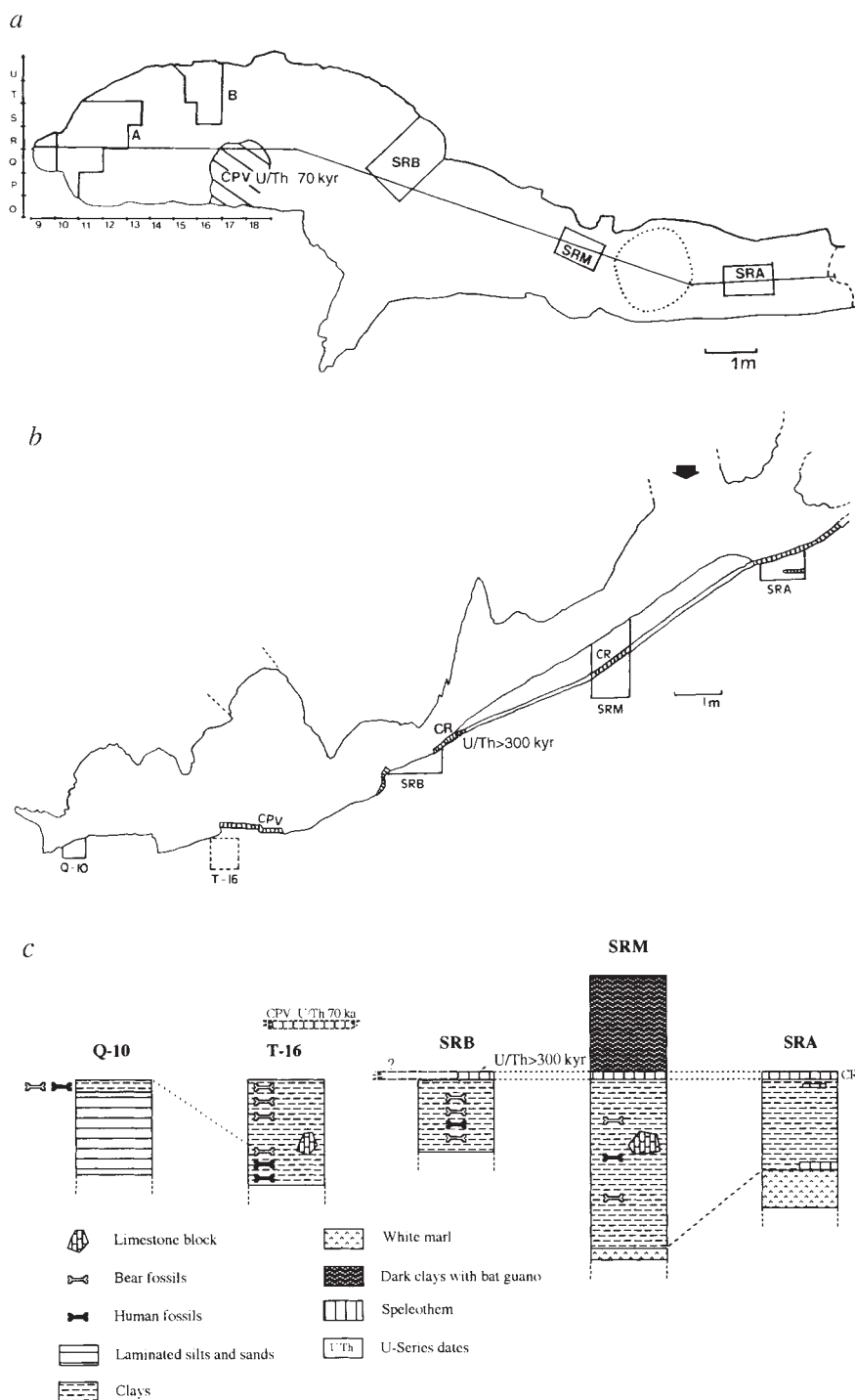


FIG. 1 *a*, Lateral and *b* superior view of cranium 4. *c*, Facial, *d*, lateral and *e*, posterior view of cranium 5. Cranium 5 preserves the right I¹, right M²–M³ and left M¹–M³, all of them heavily worn. *f*, Lateral view of cranium 6. Cranium 6 preserves most of the braincase and the face, including left upper P³–M² and right upper C, P³, M¹. The second molar is just erupted (J. M. Bermúdez de Castro, personal communication). There is an extensive osteitis on the left maxilla of cranium 5, affecting the alveolar process between C and M¹ as well as the left nasal margin. There is an unusual growth of the tympanic bone on both sides of cranium 4, that almost obliterates the external auditory meatus. As has been described in other specimens of the Atapuerca sample²⁶, the three new skulls show pathological alteration of the anterior wall of the glenoid cavity.

FIG. 2 Map and sections of Sima de los Huesos. *a*, Plan view of main excavation areas. *b*, Projected section along polygonal line in *a*. *c*, Stratigraphy and correlations. The Sima de los Huesos is more than 500 m from the entrance of the Cueva Mayor karst system, although geophysical studies from the surface suggests a nearby ancient entrance, now collapsed and filled (J. Bergamin, personal communication). Access to the site is through a 13 m shaft (arrow in *b*). CR is a continuous stalagmitic floor extending from SRA to SRB, mostly covered by sterile dark clays rich in bat guano. Bear fossils and five human fossils were found in SRM below CR and overlying sterile white marl (*c*). In SRB there is a major accumulation of bear fossils (*Ursus deningeri*), some articulated below CR crust, together with human fossils. Human fossils (including cranium 4, cranium 5 and cranium 6) were found in excavation area B, together with some bear bones (*U. deningeri*) and below a bone breccia containing only carnivore bones. Other human fossils were found in area A (with bear fossils) in a level of plastic clays which overlays sterile laminated silts and sands. Speleothem CPV covers the bone breccia in a limited area (squares R/O-16/18). Uranium series dating has been done on all speleothems exposed to date within the Sima (J. Bischoff, personal communication). A date of 70 kyr was obtained for the CPV speleothem, providing a minimum date. Multiple analyses of speleothem CR above the section at SRB showed full isotopic equilibrium indicating a date of >300 kyr. It is tempting to project this speleothem across to the top of area B, where the skulls were recovered, but whether it pinches out in the interval, or was mechanically removed by earlier exploitation by cavers is not clear at present. The presence of human fossils below CR, however, indicates a minimum age of 300 kyr for the human assemblage. Drawings were provided by A. I. Ortega and Grupo Espeleológico Edelweiss.



apomorphies have also been found in the Atapuerca mandibular sample^{17,18}. In the postcranial skeleton there are also many traits shared with Neanderthals, but these could be plesiomorphies¹². The juvenile specimen cranium 6 shows that the morphological pattern of the Atapuerca sample arises early in ontogeny, with the exception of the development of the mastoid processes and the facial prognathism. Apart from the Neanderthal apomorphies, the Atapuerca cranial sample displays a set of traits which are polymorphic and should therefore be used carefully in phylogenetic reconstruction¹⁹⁻²¹. The Atapuerca cranial sample departs from the condition generally observed in Asian *H. erectus*²²⁻²⁵ in several traits: more elevated cranial vault, separation of vertex from bregma, double arched supraorbital torus, less angulated occipital bone with opisthocranium not coincident

with inion, occipital torus reduced laterally, weak tympanic bone, high and rounded temporal squama. Although the lower limit of the cranial capacity range in Atapuerca (and in 'archaic *Homo sapiens*') overlaps the upper limit of the Asian *Homo erectus* sample, there is a substantial overall increase in brain size.

As a whole, Atapuerca and the European Middle Pleistocene cranial sample fit a model of local evolution with increasing frequencies of Neanderthal traits, although a more accurate chronological framework is needed to establish the evolutionary tempo.

Study of the whole Atapuerca cranial, dental, and postcranial sample (which is likely to increase with further excavations) will provide an unprecedented documentation of Middle

Pleistocene human variation, and permit more accurate phylogenetic reconstruction through establishment of polarities for many characters. □

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Heritable variation in a plumage indicator of viability in male great tits *Parus major*

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THE idea that female birds choose mates on the basis of genetic quality is a contentious issue in sexual selection^{1,2} because empirical evidence is lacking^{3,4}. Females mating with attractive males may obtain direct benefits such as high quality parental^{4–6} or breeding resources⁷, or indirect benefits such as offspring of high genotypic quality^{8–11}. This debate could be resolved if the traits associated with attractiveness in males have a high heritability, and correlate with the viability of their offspring. Here I report the results of a cross-fostering experiment in great tits in which parents raised unrelated young. This showed that the plumage trait associated with attractiveness in males was heritable, and that the viability of male offspring was correlated with the plumage traits of their putative father. These results show that females mating with attractive male great tits realize an indirect fitness advantage.

The great tit (*Parus major*) is a small passerine bird which inhabits woodlands, parks and gardens¹². During the breeding season, males are monogamous and defend a breeding territory in which they nest in holes. The plumage traits of males are highly variable and sexually dimorphic, the most conspicuous trait being the black breast-stripe^{13,16}. Males with large stripes are apparently preferred as mates by females¹⁵. To obtain unbiased estimates of the heritability of the stripe size of males requires a cross-fostering experiment which results in parents raising unrelated offspring¹⁶. Also, establishing that there is genotypic variation in the viability of offspring which correlates with male plumage traits requires that offspring are raised by

foster parents. This removes the effects on offspring viability of any correlation between the quality of paternal care and the plumage traits of the male^{4,6}.

The cross-fostering experiment was done on great tits nesting in nest boxes in Bagley and Wytham Woods, near Oxford, during the breeding seasons of 1990 and 1991. Cross-fostering was achieved by exchanging complete, partially incubated clutches between pairs of nests ($n = 64$ nests). The nests within each pair were matched as closely as possible for clutch size and laying date before all eggs in each clutch were exchanged. As a result, parents raised broods of similar size to their original clutch size ($r = 0.646$, $n = 58$, $P = 0.0001$) and the foster nestlings they raised hatched at a similar time in the season to their own young in another nest ($r = 0.867$, $n = 56$, $P = 0.0001$). Therefore, the cross-fostering design resulted in parents raising young unrelated to themselves, but experiencing no significant change in their breeding circumstances. The stripe size of male offspring can then be compared with the stripe size of their putative and foster

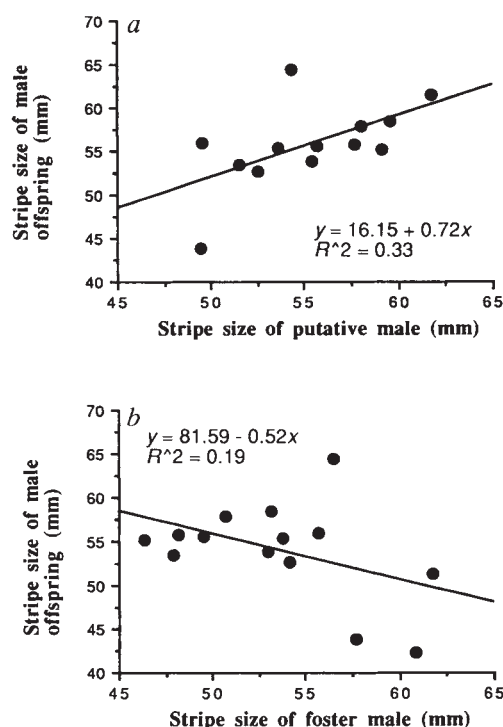


FIG. 1 Correlations between the stripe size of male offspring and the stripe size of *a*, their putative father and *b*, their foster father. The heritability corresponds to twice the slope of these regressions¹⁶. In the regression equation, x is stripe size, and R^2 is the coefficient of determination. Young great tits only moult into their adult plumage in autumn, after they have fledged¹⁷. So these analyses were based on recaptures of male offspring in the study area subsequent to their first moult. Mean stripe sizes were calculated when more than one offspring were captured from the same brood. Nestlings were ringed before fledging. Great tit parents were trapped while feeding young. The width of the stripe of males was measured to the nearest 0.1 mm at four standard points along its length, and the values summed to give a measure of stripe size. This method provides a comparable measure to the methods used to measure stripe size reported previously ($r = 0.85$, $n = 32$, $P < 0.001$)¹⁵. The stripe size of female parents was measured to the nearest 0.1 mm at the midpoint between the base of the sternum and the anterior end of the stripe because females moult their lower breast feather when forming an incubation patch before their capture. Recaptures of known males and females during subsequent breeding seasons showed that measurements made on the same individual were similar (males: $r = 0.812$, $n = 18$, $P < 0.001$; females: $r = 0.64$, $n = 25$, $P < 0.001$). The slopes of these regressions were not influenced by positive assortative mating¹⁶. There was no significant correlation between the stripe sizes of mated males and their mates in either year (1990: $r = 0.07$, $n = 20$, $P = 0.75$; 1991: $r = -0.21$, $n = 31$, $P = 0.25$).

fathers, which have both experienced similar experimental manipulations (both are raising foster clutches).

There was a significant correlation between the stripe size of cross-fostered male offspring and the stripe size of their putative father (Fig. 1a). This regression produced a heritability (h^2) estimate of $h^2 = 1.44 \pm 0.62$ ($\pm$ s.e.) ($P = 0.039$). This estimate is higher than the maximum heritability of $h^2 = 1$, but not significantly different from this value. The heritability was also estimated using a weighted analysis¹⁶, in which the mean stripe size of male offspring from a brood was weighted by the number of males recaptured from that brood. This analysis reduces the influence of any outlying points based on single observations. This analysis produced a comparable heritability estimate of $h^2 = 1.32 \pm 0.56$ ($P = 0.04$). These analyses show that the stripe size phenotype of male great tits has at least a moderate genotypic component. There was no evidence of a significant correlation between the stripe size of male offspring and the stripe size of their foster father ($h^2 = -1.04 \pm 0.62$) ($P = 0.12$) (Fig. 1b). This shows that there is no evidence to suggest that stripe size genotypes are correlated with environmental effects that influence the stripe size of male offspring. This confirms the results of the putative father regression, suggesting that stripe size has at least a moderate genetic basis.

If stripe size acts as a viability indicator, then males with large stripes should produce offspring that have high viability and so improved survival prospects^{10,11}. To test this hypothesis, the number of juvenile great tits per brood surviving to independence was tested for correlation with the stripe sizes of the putative and foster fathers. Rank correlations were used because the number of surviving offspring was strongly positively skewed: most nests produce no surviving offspring. Separate analyses were conducted on offspring of each sex, because sex influences survival and dispersal probabilities, with female great tits suffering higher mortality¹⁸ and being more likely to disperse¹⁹. There were no significant correlations between the stripe size of the foster male and the number of male or female offspring surviving from the brood he raised (Fig. 2a) (male offspring: $r = -0.089$, $P = 0.56$; female offspring: $r = 0.044$, $P = 0.77$; $N = 45$ in both cases). But there was a significant correlation between the stripe size of the putative father and the number of male offspring surviving from his fostered brood (Fig. 2b) ($r = 0.493$, $P = 0.0035$, $N = 36$). It could be argued that putative males with large stripes produce more surviving sons because their mates lay larger clutches, and so produce more fledglings. But there was no evidence to suggest that brood size and the stripe size of the putative father were correlated ($r = 0.264$, $P = 0.12$, $N = 36$). Furthermore, there was a significant rank correlation between the stripe size of the putative father and the proportion of male offspring surviving from a brood ($r = 0.482$, $P = 0.0044$, $N = 36$), showing that proportionately more males survive if their genetic father has a large stripe. There was no significant relationship between the number of surviving female offspring and the stripe size of their putative father ($r = 0.073$, $P = 0.667$, $N = 36$). This latter result is surprising because any viability differences associated with the stripe size of the putative father should be apparent in offspring of either sex. But females survive slightly less well than males¹⁸, and are also more likely to disperse from their natal area¹⁹. Consequently, a combination of higher mortality and greater dispersal in females may mask any correlation between viability in females and the plumage traits of their putative father by reducing recapture probabilities within the study areas for this sex: 50% fewer females than males were recaptured in the study areas.

These analyses suggest that the stripe size of a male great tit indicates the viability of his offspring, and is based on genotypic difference between males. But the heritability and viability correlations with the stripe size of the putative father could be influenced by maternal effects acting before the clutches were exchanged. For example, females mated to males with large stripes could lay earlier or lay larger eggs which produce

offspring that are more likely to survive and develop larger stripes. Previous work showed that there was no evidence that females laid larger eggs when mated with males with large stripes⁶. Egg size influences the mass of nestlings when they hatch, but there was no significant correlation between the stripe size of the putative father and the mean mass of his nestlings in their foster nest at hatching ($r = 0.043$, $n = 40$, $P = 0.79$) in the cross-fostering experiment. Furthermore, there was no evidence that the clutch laid by females mated to males with a large stripe were likely to hatch earlier in the season ($r = 0.024$, $n = 43$, $P = 0.88$). These analyses suggest that maternal effects are unlikely to confound the heritability and viability analyses.

The viability analysis (see Fig. 2) could be influenced by dispersal. For example, the offspring from males with small stripes may be more likely to disperse, reducing apparent survival within the study areas. Even if this process occurred, dispersing males are likely to show reduced survival because immigrant males in great tit populations survive less well than residents (males that breed in the same population in which they were hatched)¹⁸. In addition, if offspring from males with small stripes are more likely to disperse, then this suggests that immigrant males to a population should have smaller stripes than resident males. This was tested by comparing the stripe size of males of known immigration status in the Wytham population because the status of all males was only accurately known at this site. This analysis showed no difference between the stripe sizes of immigrant and resident males (immigrant males: 53.0 ± 1.5 mm; resident males: 53.9 ± 1.1 mm; $t = 0.48$; $n = 31$, $P = 0.63$). This suggests that dispersal is unlikely to account for the higher viability shown by male offspring from putative males with large stripes.

The results of the cross-fostering experiment show that females mating with males with large stripes realize indirect fitness benefits by improving the genotypic viability of their

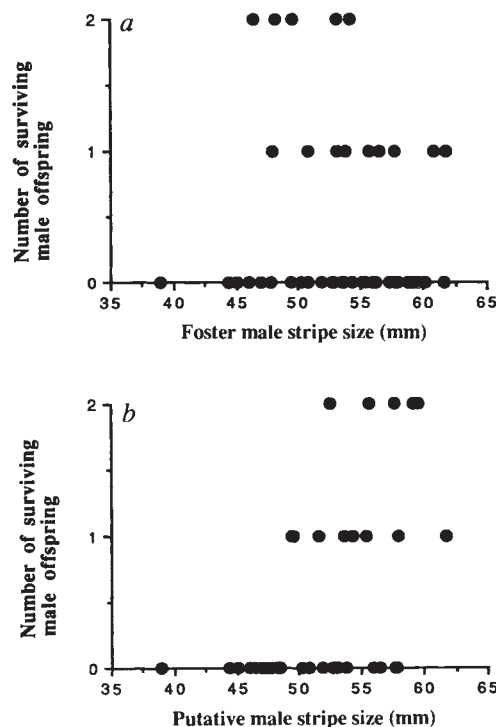


FIG. 2 The viability of juvenile male great tits in relation to a, the stripe size of their foster father and b, the stripe size of their putative father. The viability of juveniles corresponds to the number of independent offspring from a cross-fostered brood which were recaptured during a subsequent winter or breeding season in the study areas.

offspring. Other studies have documented significant heritability in plumage traits preferred by female birds, suggesting a role for indirect benefits for females in other species^{4,20,21}. But it is possible that these estimates could be biased because of correlations or interactions between parental genotypes and the environment. The results of my cross-fostering experiment do not preclude the role of direct benefits⁶, but do indicate that indirect fitness effects are also important in the evolution of female preferences in great tits. The mechanism underlying genotypic quality variation and its maintenance is unknown at present, although a number of potential mechanisms are known^{3,10,22,23}. □

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Is pattern vision in insects mediated by 'cortical' processing?

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It is known that bees, like humans, can learn the orientation of a striped pattern, and recognize this orientation in other simple patterns that they have never previously encountered^{1,2}. How is orientation analysed by the insect visual system? In the light of what is known about animal vision, there are two obvious possibilities. First, orientation could be determined purely in terms of the directional movement signals that the pattern generates as the bee approaches it or flies past it^{3,4}. Such a scheme would fit in well with the common supposition that much of insect vision relies solely on motion cues^{4,5}. An alternative view, not considered for insect vision so far, would be that specific features of the pattern, such as bars or edges, are extracted and their orientation analysed as in the mammalian cortex. Our findings argue strongly against the first and for the second possibility. They suggest that similar principles underlie the analysis of pattern orientation in insects and higher vertebrates.

In behavioural experiments, we examined whether honey-bees (*Apis mellifera*) discriminate the orientation of a pattern on the basis of (1) the motion cues generated as the insect flies past the pattern, or (2) the geometrical orientation of the pattern's constituent features. Bees were trained in a Y-maze to distinguish between stationary random patterns of horizontal and vertical stripes (Fig. 1a). The trained bees were unable to distin-

guish between random dot patterns (Fig. 1b). But when the dots were rearranged to form horizontal and vertical rows, the same bees showed a strong preference for the pattern with the rewarded orientation (Fig. 1c). They must therefore see the global pattern in the arrangement of the dots. In agreement with earlier results^{1,2}, these experiments demonstrate that bees trained to a particular orientation are able to recognize this orientation in novel patterns.

Is orientation analysed through motion cues? The animal's self-motion as it approaches a pattern would generate image motion that is predominantly vertical or horizontal, according to whether the pattern is composed of horizontal or vertical stripes. This would be a simple means of inferring orientation, without extracting features^{3,4}. Such a hypothesis is an attractive one for insects, given that their visual systems are highly sensitive to movement, and contain groups of neurons that respond selectively to upward, downward, leftward or rightward motion⁶.

We found, however, that the bees trained to distinguish between horizontal and vertical stripes failed to distinguish between random-dot patterns moving horizontally and vertically (Fig. 1d). By contrast, the same bees discriminated moving row patterns (Fig. 1e) just as well as the stationary row patterns (Fig. 1c), despite the fact that the motion of each moving row pattern signalled an orientation that conflicted with the pattern's geometrical orientation. In another set of experiments we

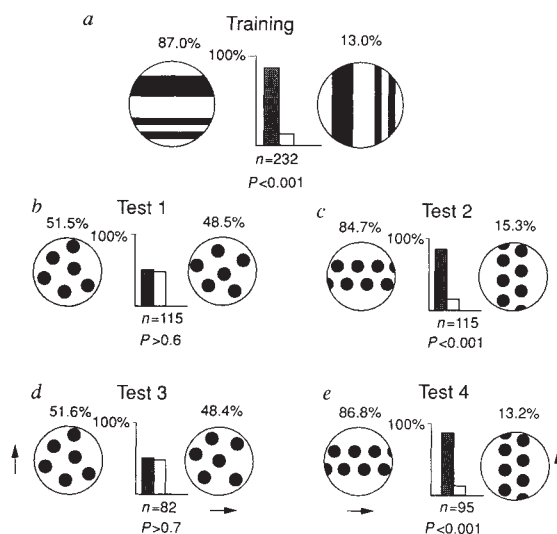
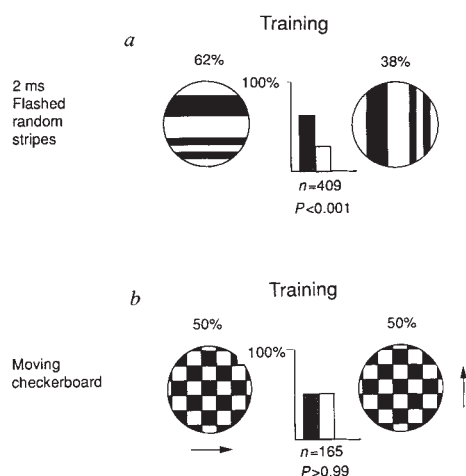


FIG. 1 Experiments investigating the role of motion cues in discriminating pattern orientation. Each panel shows a pair of stimuli used in training or in subsequent tests, the bees' choice frequencies for the two stimuli, the number of choices analysed (n), and the P value in a χ^2 test for a significant departure from random choice. Bees can be readily trained by reward in a Y-maze to distinguish between horizontally and vertically oriented patterns. The training procedure, which uses a large number of pairs of randomly striped, stationary patterns, ensures that the bees learn orientation irrespective of pattern¹. Bees trained in this way learn to discriminate the orientation of striped patterns well (a). These trained bees do not distinguish between random-dot patterns either when the patterns are stationary (b) or when one moves vertically and the other horizontally (d). But patterns composed of horizontal or vertical rows of dots are readily distinguished, regardless of whether they are stationary (c) or in motion (e). Thus orientation discrimination depends solely on pattern geometry. It is neither reliant upon motion cues, nor affected by motion. All stimuli had a diameter of 24.5 cm. The training patterns were composed of black and white stripes whose widths were random multiples of 2.0 cm. The dots had a diameter of 4.8 cm. In the random-dot patterns, the centre-to-centre separation of the dots varied between 5.5 cm and 14.0 cm. In the row patterns the separation was 5.5 cm within a row, and 8.5 cm between rows. Motion was at 10 cm s⁻¹. Individual elements of the patterns (bars or dots) could be clearly resolved³ at the distance of 27 cm from which the bees choose between the two stimuli in the Y-maze.



showed that bees, trained to discriminate between random patterns of horizontal and vertical stripes as in Fig. 1a, but now displayed electronically on a cathode ray oscilloscope, continue to discriminate orientation well when they are tested by presenting the patterns for very brief durations (25 ms every 0.5 s). The trained bees showed a choice frequency of 66% for the rewarded orientation ($P < 0.001$, $n = 295$, χ^2 test; data not shown). In a further experiment, we trained bees to distinguish between briefly presented random horizontal and vertical stripes and found that orientation was discriminated even when the duration of each presentation was as low as 2 ms (Fig. 2a). It is extremely unlikely that motion cues were extracted during such brief presentations. Finally, if bees use directional motion cues to infer pattern orientation, one might expect to be able to train them to distinguish between patterns which move in different directions, but present no differences in orientation. We found, however, that bees could not be trained to distinguish between identical checkerboard patterns moving vertically and horizontally, even after two days' training during which individuals were rewarded over 60 times (Fig. 2b).

These findings demonstrate that the orientation of a pattern is discriminated on the basis of geometrical cues, and not by cues derived from image motion. They suggest the existence of orientation channels analogous in mechanism to those in the mammalian visual cortex. Our results do not imply that orientation analysis by the bee's visual system does not require any image motion at all: as in human vision, temporal fluctuations of intensity (as induced by motion of flicker) may be necessary to ensure that the image remains visible^{7,8}. It is clear from our data, however, that the perception of stripe orientation does not involve an analysis of image motion.

FIG. 2 Bees can be trained to distinguish between random patterns of horizontal and vertical stripes, even when such patterns are presented for very brief durations, as in a, when presentation was for 2 ms every 0.5 s. Thus, orientation discrimination persists even when image motion cues are eliminated. Each brief presentation displayed a different pair of randomly striped patterns, cancelling any apparent-motion cues that could have been obtained by comparing the image of the pattern in one presentation with the afterimage induced in the eye by the previous presentation. Further evidence that directional motion cues are not relevant to orientation discrimination comes from the finding that bees cannot be trained to distinguish between patterns which move in different directions, but present no differences in orientation, as in b. The checkerboard patterns were composed of 4.0 cm squares, moving at 50 cm s⁻¹ and thus producing a contrast frequency of 6.5 Hz in the eye of a stationary bee. These stimuli were presented on a computer-controlled CRT display with a frame rate of 200 Hz, which is beyond the flicker fusion frequency of the bee's visual system¹⁷. Thus the stimuli in b would have been flicker-free for the bees. Each flash of the stimuli in a consisted of one 500-line frame which took 2.0 ms to plot on the CRT screen. The decay time of the phosphor was measured to be 1.0 ms. The vertical stripes were plotted on a vertically oriented raster, and the horizontal stripes on a horizontally oriented raster. To control for the (very unlikely) possibility that the bees were inferring the orientation of the stripes of the stimuli in a by discriminating the directions in which the rasters were plotted, we attempted in a separate experiment to train bees to distinguish between two uniform-intensity fields, one created on a horizontal raster and the other on a vertical raster. The bees were unable to discriminate between the two stimuli ($P > 0.95$, $n = 251$), showing that the direction of plot of the raster had no influence on their discrimination performance.

In primates, cortical physiology suggests that orientation within a patch of visual space is analysed by a system of about 18 orientation-sensitive channels, with preferred orientations separated by about 10 degrees⁹. Our behavioural data do not necessarily imply, or require, that insects possess a system of comparable architecture or complexity. In principle, three broadly tuned orientation-sensitive channels with different preferred orientations, would suffice to provide unambiguous orientation information within a given patch of visual space. The hexagonal arrangement of the ommatidia in the insect compound eye is well suited to the organization of channels with preferred orientations separated by 120°. Likely sites of the putative orientation-sensitive neurons in the insect visual pathway would be the medulla or the lobula, neither of which has been explored extensively. Both of these neuropils are organized in retinotopic columns, with most of the neurons in each column viewing a small patch of visual space, not unlike the situation in a cortical hypercolumn. Indeed, direction-insensitive, orientation-tuned units have recently been encountered in the lobula of the dragonfly¹⁰.

Much of the earlier work on pattern recognition in insects has highlighted pattern-discrimination mechanisms that are based on an analysis of structural complexity¹¹ or on a 'photographic' memorization of patterns¹²⁻¹⁴. Our results suggest, perhaps for the first time, the existence of feature-extracting mechanisms in the insect visual system that might be comparable, functionally, to those known to exist in the mammalian cortex. The recent finding that insects perceive illusory contours^{1,15} reinforces the possibility that insect vision may well embody some of the computational principles of area 18 in primate cortex¹⁶. □

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Feature-detecting neurons in dragonflies

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SINCE the earliest descriptions¹ of the compound eye, the popular impression has prevailed that insects and mammals view the world differently. Recent work, however, underscores marked evolutionary convergence between the visual systems of vertebrates and insects at both optical^{2,3} and early processing levels^{4,5}. Here I describe several classes of cells from the third optic ganglion of dragonflies that respond selectively to different target classes. Several physiological properties of these cells are remarkably similar to those of cells from areas 17, 18 and 19 of the mammalian visual cortex. One class of bar-sensitive, orientation-biased cells could mediate discrimination of the orientation of low spatial frequency components of patterns. The existence of neurons functionally similar in many respects to those in the mammalian cortex suggests that evolutionary convergence in visual processing is not limited to early pathways. Insects, like mammals, seem to possess mechanisms for extracting spatial features from visual scenes.

There is no 'high order' visual structure in the insect nervous system that is equivalent to the mammalian cortex. Although the optic lobes comprise three ganglia with a highly parallel, retinotopic organization^{6,7}, there is no evidence for elaborate columnar organizations of cells with a complexity equivalent to that which characterizes the mammalian cortex⁸. Nevertheless, it is possible that neurons in the second and third optic ganglia (the medulla and lobula complex) integrate visual information

across the retinotopic array in a manner embodying some of the principles of processing by individual cell types of the cortex.

Much work on visual processing by the insect optic lobes has concentrated on cells from a specialized region of the lobula (the lobula plate), believed to mediate the optomotor response^{5,9}. Other cell classes have been described, including units that respond selectively to targets moving relative to background¹⁰⁻¹³ and cells that respond to changes in intensity¹⁴, but the physiology of most cells in the medulla and lobula remains unknown.

Recording intracellularly from the lobula complex of dragonflies (*Hemicordulia tau*), I have characterized over 150 cells that respond to targets of various sizes, belonging to at least 26 anatomical cell classes (D.O.C., manuscript in preparation). Physiologically these cells can be divided into two general groups on the basis of the preferred stimulus, termed here 'small target-sensitive' and 'bar-sensitive' cells.

Small target-sensitive cells respond selectively to small moving targets. Some have excitatory receptive fields with a half-width subtending less than 10° (Fig. 1). Small targets or bars moved within this region elicit strong responses, but larger stimuli moved across the centre of the receptive field elicit no response (Fig. 1). Other units have larger receptive fields, in some cases extending across the visual field of the eye. All of these cells are sharply tuned to the motion of dark targets subtending visual angles equivalent to just one or two facets of the compound eye (about 1-2°): wide-field motion (of gratings or large bars, for example) elicits no response (Fig. 2, left; Fig. 3). No other cells

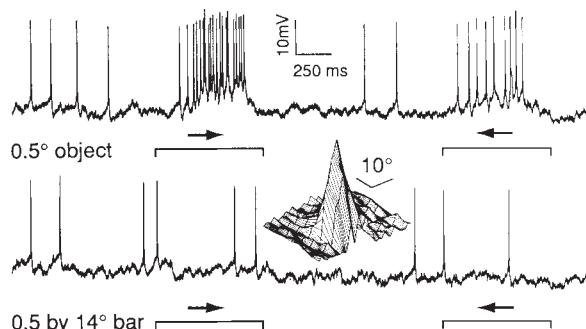


FIG. 1 Intracellular potentials recorded from a small receptive field, small target-sensitive unit from the lobula (third optic ganglion) of a dragonfly. The cell gives a strong spiking response superimposed on noisy graded potentials when a small target is moved through its receptive field (upper trace). The weaker response to motion in the opposite direction (right) illustrates a direction selectivity characteristic of these cells (Fig. 4). When the stimulus is extended orthogonally into a bar (lower trace), the response is inhibited. The response period of ~500 ms in the upper trace corresponds to an excitatory receptive field size of 8°. The inset shows a three-dimensional reconstruction of the receptive field shape for a unit with a slightly broader receptive field. Sequential 'scans' of the target at 4° intervals across the stimulus screen were used to generate data for the three-dimensional plot. The instantaneous spike rate was calculated from peri-stimulus time histograms from 5 presentations at each location in the receptive field. Recordings were made with aluminium silicate micropipettes introduced into the optic lobe through a small hole in the posterior head capsule. Stimuli generated by a Picasso image synthesizer were presented to the insect on a high intensity xyz display (background luminance ~30 Cd m⁻²) at frame rates of ~200 Hz. The target was moved at 17° per s for a period of 750 ms first in one direction then in the opposite direction after a 1,250 ms rest period (as indicated by the bars).

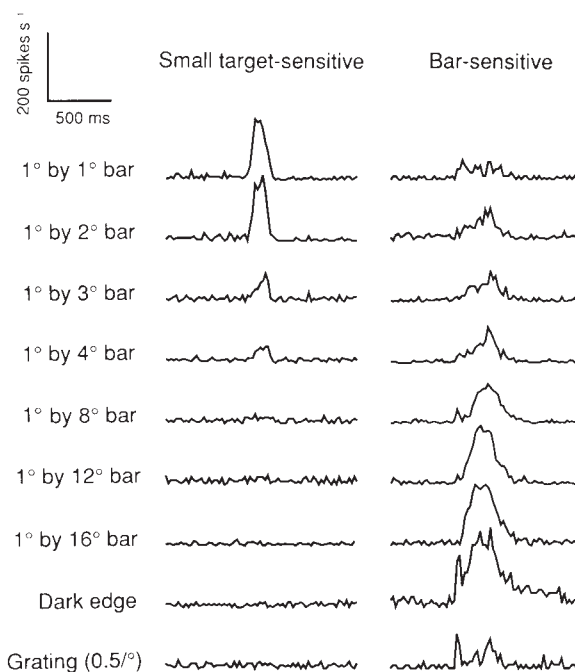


FIG. 2 Peri-stimulus time histograms from a large receptive field, small target-sensitive unit (left) and a bar-sensitive unit (right). Small target-sensitive units respond very strongly to targets subtending visual angles of 1-2° but show a sharp inhibition as the stimulus is extended into a bar, and give no response to large-field stimuli such as gratings or a single dark edge (lower). By contrast, bar-sensitive units show spatial summation, giving larger responses as the angular subtense of a bar stimulus is increased (right), and give the best response to a single bar or edge. Nevertheless, a much weaker response results from a square-wave grating with the same spatial wavelength as the optimal bars (lower), implying the presence of some form of spatial inhibition. Intracellular potentials were digitized at a sample frequency of 5 kHz and thresholded to permit detection of spike arrival times. Histograms were then constructed from the spiking component of the response. Data shown are in 20-ms bins and are the signal average of 10 successive stimulus presentations, with a 10-s rest period between presentations.

reported from insect optic lobes⁹⁻¹³ show such a sharp and well defined spatial tuning of response. For example, the so-called figure-detection cells described from the lobula plate of the fly^{9,13} give maximal responses to moving patterns subtending 15–20° and still give half-maximal responses to patterns as large as 50°.

Bar-sensitive cells are also unlike any cells previously described from insects. Like tangential cells of the lobula plate⁹, they give larger responses as the stimulus size is increased (Fig. 2). Unlike the latter, however, these units give only weak responses to wide-field moving gratings except at very low spatial frequencies. The best response is to a single moving edge or bar, either light or dark, and the response to a single bar is much stronger than to a grating composed of identical bars (Fig. 2, right). The spatial gain is also much higher than for other cells described. For example, the half-maximal response is obtained at a stimulus area of 5 square degrees (Fig. 3) compared with 500 square degrees for the HSE cell of the lobula plate⁹.

The preferred stimulus size and the shape of stimulus length/response curves for small target-sensitive cells (Fig. 3) are essentially identical to those published for hypercomplex I (S_H) cells in the cat striate cortex^{8,15,16}. Stimulus length/response curves for bar-sensitive cells are remarkably similar to those for simple and complex cells in the cat striate cortex^{8,15,16} (Fig. 3). Most small target-sensitive cells are direction-selective, characterized by a single preferred direction for stimulus motion (Figs 1, 4). By contrast, bar-sensitive units are not direction-selective but rather show a preference for a correctly oriented bar or edge moved in either direction across their receptive field (Fig. 4). This 'orientation tuning' is similar to that seen in many cells from areas 17, 18 and 19 of the cat visual cortex¹⁶⁻¹⁸. The tuning

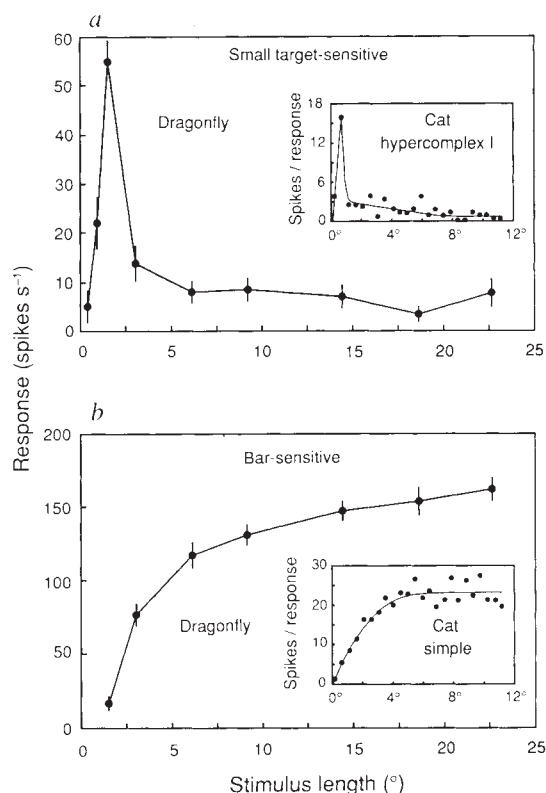


FIG. 3 Stimulus length/response curves for cells from the lobula of dragonflies. The insets in each case show similar plots for cells from the cat striate cortex (adapted from ref. 15). There is a striking similarity between the curve for dragonfly small target-sensitive cells (a) and 'end-stopped' simple (hypercomplex I) cells of the cat (inset), and between that for a bar-sensitive cell (b) and 'end-free' simple cells (inset). Data are taken from peri-stimulus time histograms and are the average response ($\pm$ s.e.) from 10 presentations (with a 10-s rest period between presentations) of 1° wide dark stimuli.

half-width (at $\text{response}_{\max} - \text{response}_{\min}/2$) of $\sim 90^\circ$, although broader than that of most cells in areas 17 and 18 of cat cortex, is close to the median value of cells in area 19 (ref. 8).

Small target- and bar-sensitive cells give little or no response to either wide-field or point source flicker. As is the case with many cells of the cortex^{8,16}, responses are only evoked as a target is moved across the excitatory receptive field. Unfortunately, the moving bar stimulus that must be used to quantify the orientation selectivity of bar-sensitive cells confounds the stimulus orientation with directional motion cues, contaminating the response^{19,20}. The orientation and directional components of the confounded response are periodic functions with a period of 180° and 360°, respectively^{19,20}. Recent analyses of such data from the cortex have attempted to 'unconfound' the

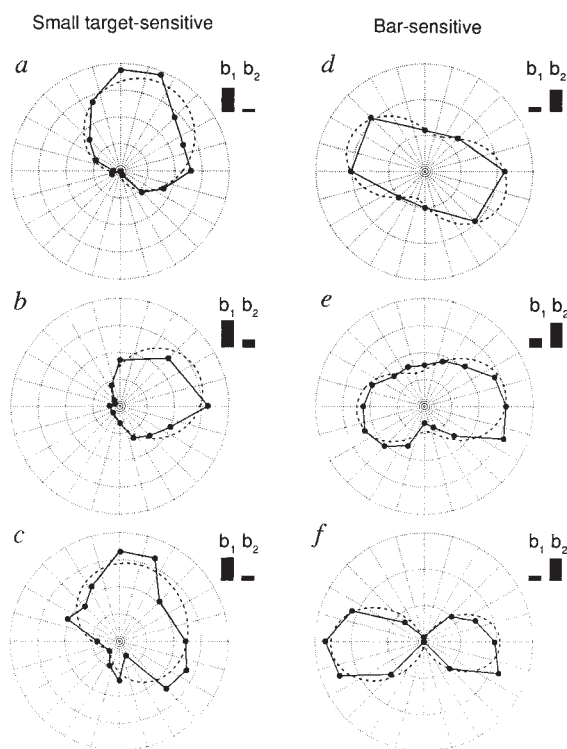


FIG. 4 Polar plots showing the mean response from 6 cells for moving stimuli presented along different axes. Most small target-sensitive units are direction-selective (left; a, b, c) and have a single preferred direction of stimulus motion, in this case for upward motion (a, c) and rightward motion (b). Bar-sensitive cells (right; d, e, f) include units with no single preferred direction but rather a bias for bars moved in either direction horizontally across their receptive field. These cells, like cells in areas 17, 18 and 19 of the cat visual cortex^{8,16-18}, are thus tuned to bars with an orientation perpendicular to the direction of the lobes on the plot. The dashed lines show the results of Fourier decomposition of the data by regression onto a model of the general form $R(\theta) = b_0 + b_1 \sin(\theta + \phi_1) + b_2 \sin(2\theta + \phi_2)$, where R is the response at direction θ , b_1 and b_2 are the coefficient and ϕ_1 and ϕ_2 are the phase for the direction and orientation components of the response, permitting the directional and orientation components of the response to be 'unconfounded' (see text). Bar graphs adjacent to each plot show the root-mean-square power of the directional (b_1) and orientation (b_2) components, normalized to the sum of both. In the case of bar-sensitive cells (right), b_2 is much larger than b_1 , showing that most of the response is due to the stimulus orientation, not the direction of motion. Data shown have the spontaneous activity subtracted from the response. All data are taken from peristimulus time histograms and are the average response from 10 presentations in each direction, presented in random order. The distance over which targets were moved was restricted to a small region ($\sim 10^\circ$) of the overall receptive field ($\sim 100^\circ$). The stimulus contrast was kept low ($\Delta I/I_{\text{background}} = 0.1$), which gave roughly half-maximal responses at the preferred direction/orientation.

two components using Fourier decomposition^{19,20}. A similar analysis of the data for the bar-sensitive cells described here (Fig. 4) shows conclusively that the response component with a period of 180° comprises the majority of the response. The cells thus respond primarily to the stimulus orientation and not to the direction of motion (Fig. 4).

Although the results described here provide no evidence for a direct analogy between the retinotopic organization of the insect lobula and the mammalian cortex, the functional similarity between the spatial properties and orientation tuning of cells from the two groups prompts some speculation as to the significance of the apparent convergence. Feature detection seems a likely role for pathways with properties such as target specificity and orientation tuning, and whereas it is simplistic to regard the mammalian visual cortex as an array of feature detectors⁸, the small number of cells in the insect visual system (<10⁶ in total⁶) makes the existence of hard-wired feature detectors an attractive hypothesis.

Honey-bees can learn the orientation of a pattern and recognize this orientation in even novel patterns^{21,22}. While honey-bees and dragonflies are not closely related, the present finding of orientation-tuned cells apparently selective for low spatial frequency components of patterns at least provides a neural substrate for such behaviour.

Direction-selective, small target-sensitive cells most probably form part of an unrelated pathway used for the detection of small moving targets such as conspecifics or (in the case of predatory insects like dragonflies) prey. The outputs of small receptive field cells, such as those described, could be integrated by cells, such as those described, with larger receptive fields.

These in turn could connect to the descending 'target' interneurons described from the ventral nerve cord of dragonflies^{23,24}. □

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Neurotoxicity of a prion protein fragment

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THE cellular prion protein (PrP^C) is a sialoglycoprotein of *M*_r 33–35K that is expressed predominantly in neurons^{1–3}. In transmissible and genetic neurodegenerative disorders such as scrapie of sheep, spongiform encephalopathy of cattle and Creutzfeldt–Jakob or Gerstmann–Sträussler–Scheinker diseases of humans^{4,5}, PrP^C is converted into an altered form (termed PrP^{Sc}) which is distinguishable from its normal homologue by its relative resistance to protease digestion^{6–8}. PrP^{Sc} accumulates in the central nervous system of affected individuals^{8,9}, and its protease-resistant core aggregates extracellularly into amyloid fibrils^{10–12}. The process is accompanied by nerve cell loss, whose pathogenesis and molecular basis are not understood. We report here that neuronal death results from chronic exposure of primary rat hippocampal cultures to micromolar concentrations of a peptide corresponding to residues 106–126 of the amino-acid sequence deduced from human PrP complementary DNA. DNA fragmentation of degenerating neurons indicates that cell death occurred by apoptosis. The PrP peptide 106–126 has a high intrinsic ability to polymerize into amyloid-like fibrils *in vitro*. These findings indicate that cerebral accumulation of PrP^{Sc} and its degradation products may play a role in the nerve cell degeneration that occurs in prion-related encephalopathies.

On the basis of the observation that the β -protein responsible for the extracellular deposition of amyloid fibrils in Alzheimer's disease is neurotoxic^{13,14}, we hypothesized that neuronal death

in prion-related encephalopathies might be due to abnormal extracellular accumulation of PrP^{Sc} and/or its degradation products. We therefore investigated the effects of synthetic peptides homologous to different segments of PrP on the viability of primary rat hippocampal neurons. The peptides selected for this study matched the PrP region corresponding to the amyloid protein purified from the cerebral cortex of patients of an Indiana family affected by Gerstmann–Sträussler–Scheinker

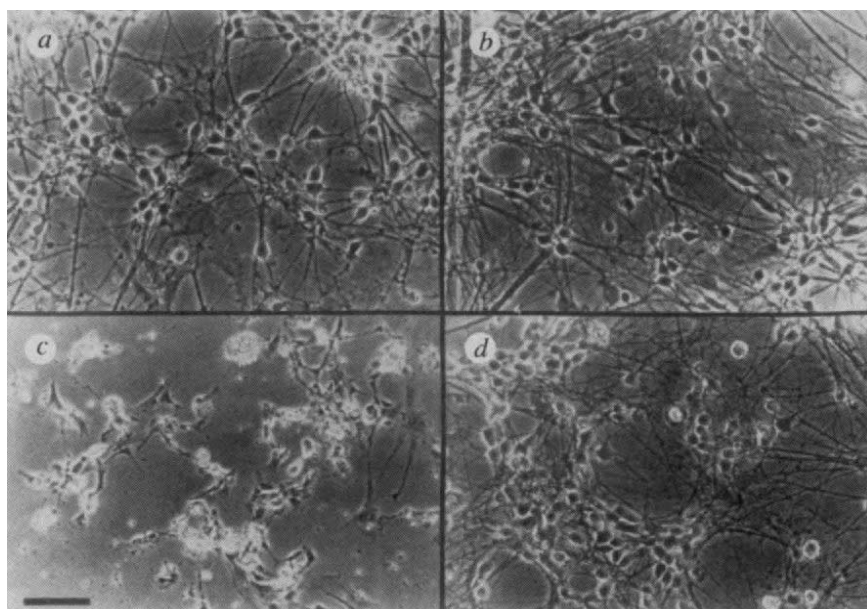


FIG. 1 Synthetic peptides used for this study (single-letter amino-acid code): a, PrP57–64, WGQPHGGG; b, PrP89–106, WGQGGGTHSQWNKPSKPK; c, PrP106–114, KTNMKHMAG; d, PrP106–126, KTNMKHMAGAAAAGAVVGGGL; e, PrP127–135, GYMLGSAMS; f, PrP127–147, GYMLGSAMSRPIHFGSDYED; g, PrP106–126 scrambled, NGAKALMGHGATKVMVGAAA; a–f, amino-acid sequence of peptides homologous to different fragments of the amyloid protein purified from GSS brains (residues 58 to ~150)¹². g, Scrambled version of PrP106–126. The octapeptide 'a' is repeated four or five times in the PrP sequence.

METHODS. Peptides were synthesized using solid-phase chemistry by means of a 430A Applied Biosystem instrument. F-moc (9-fluorenylmethoxycarbonyl) was used as the protective group for amino residues, and 1-hydroxybenzotriazole, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and *N,N*-dicyclohexyl-carbodiimide as activators of carboxylic residues. Crude peptides were purified by crystallization. Their purity and composition were determined by analytical reversed phase HPLC (Beckman), capillary electrophoresis (Millipore), amino-acid analysis (Beckman 6300 amino acid analyser) and amino-acid sequencing (Milligen 6600 prosequencer). A purity of >99% was confirmed for all peptides used. Some peptides, including PrP106–126, were further purified by reverse-phase HPLC on a C-18 column (2.0 × 25 cm, Spherisorb) to rule out the possibility that contaminants copurified with the peptides. The HPLC-purified peptides exhibited the same effect on neuronal viability of peptides purified by crystallization.

FIG. 2 Neurotoxic effect of peptide PrP106–126. Photomicrographs of hippocampal neurons after 10 days in culture: *a*, Control culture, chronically exposed to the vehicle solution; *b*, culture after acute exposure (24 h) to PrP106–126, 80 μ M; *c*, culture chronically exposed to PrP106–126, 80 μ M; *d*, culture chronically exposed to PrP106–126, scrambled, 80 μ M; scale bar, 200 μ m. Only culture chronically treated with PrP106–126 showed neuronal degeneration.

METHODS. The hippocampal neuron cultures were prepared as follows. Brains were removed from fetal rats on embryonic day 17. Hippocampal cells were dissociated in serum-free medium containing 0.1% trypsin (DIFCO) and 25 μ g ml⁻¹ deoxyribonuclease for 5 min at room temperature, and plated (5×10^5 cells per well) in Primaria (Falcon) 15-mm wells, precoated with poly-D-lysine (50 μ g ml⁻¹; Sigma). The cells were cultured in basal medium Eagle (BME-Hanks' salt, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO) and glutamine (2 mM). Cultures were kept at 37 °C in a humidified 5% CO₂ atmosphere. After 5 days *in vitro*, non-neuronal cell division was halted by exposure to 10⁻⁵ M cytosine arabinoside, an inhibitor of mitosis, to prevent overgrowth of glial cells³³. For acute treatment, peptides were applied once at the time of plating or after 9 days of culture; for chronic treatment they were added to the medium on days 0, 2, 4, 6 and 8 of culture. Cell viability was evaluated on day 10.



(GSS) disease¹² (Fig. 1). Cultures were prepared from rat hippocampus on embryonic day 17 (Fig. 2 legend) and were exposed either acutely or chronically to PrP peptides, or to a scrambled sequence of PrP106–126 used as a control (Fig. 1). For acute experiments the peptides were applied once at the time of plating or after 9 days of culture, whereas for chronic treatment they were added to the medium on days 0, 2, 4, 6 and 8 of culture. On day 10, cell viability was evaluated and compared with that of cultures treated with vehicle only. A single exposure to PrP peptides (20–100 μ M) did not affect cell survival as compared to control conditions (Fig. 2*a*, *b*). By contrast, there was great neuronal loss in hippocampal neurons chronically exposed to PrP106–126 at a concentration of 80 μ M (Figs 2*c* and 3*a*). Under the same conditions, the other PrP peptides

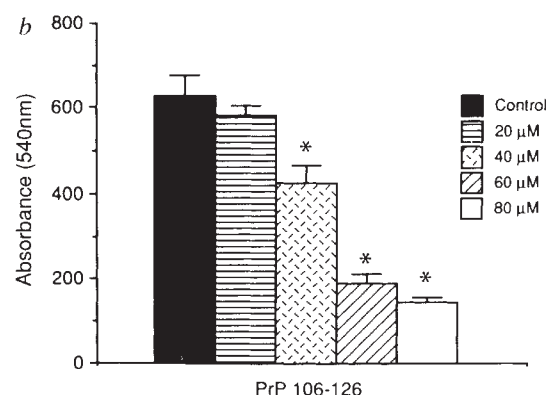
(that is, PrP57–64, 89–106, 106–114, 127–135, 127–147) and the scrambled sequence of PrP106–126 did not significantly reduce cell viability (Figs 2*d* and 3*a*). The neurotoxic effect of PrP106–126 was dose-dependent (Fig. 3*b*). The toxic response was first detected at a concentration of 20 μ M, was statistically significant at 40 μ M, and resulted in virtually complete neuronal loss at 60 or 80 μ M.

We then investigated whether a mechanism of programmed cell death was involved in the neurotoxicity induced by PrP106–126. In the nervous system, several mechanisms of programmed cell death were suggested but only apoptosis was properly described^{15,16}. Apoptotic cells have a distinct morphology, and show a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA in internucleosomal regions¹⁵.

(a) Chronic treatment of hippocampal neurons

Peptide	Neurotoxic effect	
	20 μ M	80 μ M
PrP106–126	18 $\pm$ 8	100 $\pm$ 8*
PrP57–64	0 $\pm$ 5	3 $\pm$ 4
PrP89–106	5 $\pm$ 2	2 $\pm$ 6
PrP106–114	0 $\pm$ 3	12 $\pm$ 6
PrP127–135	3 $\pm$ 6	15 $\pm$ 9
PrP127–147	1 $\pm$ 7	18 $\pm$ 7
PrP106–126 scrambled	3 $\pm$ 2	8 $\pm$ 3

FIG. 3 Quantitative evaluation of the toxic effect of PrP peptides. *a*, Hippocampal neurons were chronically treated as described in Fig. 2. The data are the mean $\pm$ s.e. of 6–10 determinations and are normalized to the toxic effect of PrP106–126 (designated 100% response). * P < 0.01 versus control group (Dunnett's test) before normalization of data. *b*, Neurotoxicity of PrP106–126 as a function of peptide concentration. The toxic response was determined after 10 days of chronic exposure of cells. Data are the mean $\pm$ s.e., * P < 0.01 versus control group (Dunnett's test).



METHODS. Neuronal cell death was quantitatively assessed by crystal violet staining (0.5% in water/methanol, 4:1) of cells cultured in Microtiter dishes. After washing, cells were dried, solubilized in sodium citrate/ethanol 1:1 and spectrophotometrically analysed at 540 nm with an automated microplate reader (Perkin-Elmer lambda reader)³⁴. The absorbance of the solution is proportional to the number of cells. Preliminary studies showed that neuronal survival as evaluated by this method corresponded with neuronal viability determinations using the dye Trypan blue.

Morphological examination of nerve cell nuclei, stained with DNA-binding fluorochrome Hoechst 33258, showed that several hippocampal neurons chronically exposed to PrP106–126 presented a typical apoptotic morphology, with condensation of chromatin and fragmentation of the nucleus (Fig. 4a). Agarose gel electrophoretic analysis of DNA extracted from cultured cells after a 7-day treatment with PrP106–126 revealed DNA fragmentation similar to that observed in promyelocytic HL-60 cells treated with actinomycin D, used as a positive control¹⁷ (Fig. 4b). Neurons exposed to the other PrP peptides (including scrambled 106–126) presented a minimal DNA fragmentation which was comparable to that of vehicle-treated cells. Small fragmented DNA was separated by centrifugation and quantified by spectrophotometry¹⁸. DNA extracted from control hippocampal cells cultured for 10 days showed a low degree of fragmentation, whereas a substantial proportion of total DNA derived from PrP106–126-treated cells was fragmented (percentage of total DNA (means $\pm$ s.e., $n = 6$): control = 7 ± 2 , PrP106–126 = 21 ± 4 ; $P < 0.01$, Student's t -test). This difference is remarkable because neurodegeneration was produced by chronic exposure and the onset of apoptosis in individual members of a neuronal population appeared to be asynchronous. Thus, morphological and biochemical features of apoptosis marked neuronal death induced by PrP fragments.

Although the results obtained *in vivo* are still controversial^{19–21}, peptides homologous to the β -amyloid of Alzheimer's disease do possess neurotoxic activity *in vitro*^{13,22}. The neurotoxicity of β -peptides has been attributed to their self-aggregation tendency, leading to formation of amyloid-like fibrils²². To investigate whether a similar property was associated to the toxic effect of peptide PrP106–126, we analysed its inherent fibrillogenic ability. The peptide was sparingly soluble in distilled water and readily formed a dense meshwork of straight

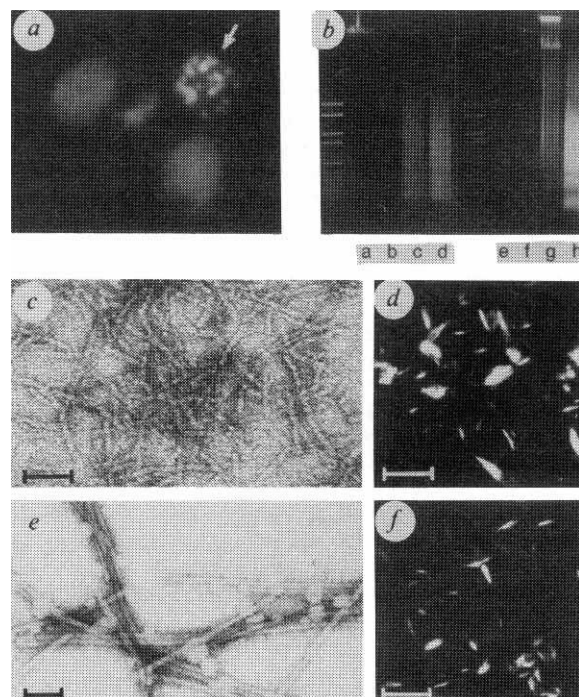
filaments. Individual fibrils had a diameter of 4–8 nm and ranged in length from 0.1 μ m to 1 μ m (Fig. 4c). When stained with Congo red, the peptide aggregates showed green birefringence under polarized light (Fig. 4d), indicative of the β -sheet conformation characteristic of amyloid. Under the same conditions, the other peptides (including the scrambled version of PrP106–126) were not fibrillogenic, with the exception of PrP106–114 and PrP127–147, which showed a moderate ability to assemble into filaments (Fig. 4e, f). Meshworks of these filaments were less dense than those formed by PrP106–126 (ref. 23; see Fig. 4c, e for comparison). The absence of toxic effects of PrP106–114 and 127–147 (Fig. 3a) indicates that the fibrillogenic properties alone are not sufficient for neurotoxicity.

In this study, the chronic exposure of rat hippocampal neurons to a PrP peptide induced dose-dependent neuronal death by an apoptotic mechanism. Our model may mimic the pathological condition that occurs *in vivo* in prion-related encephalopathies. In the terminal stages of subacute encephalopathies, such as scrapie, PrP^{Sc} reaches a whole brain concentration 10 to 20 times greater than PrP^C (ref. 9). Further, because the abnormal PrP isoform is concentrated in the neuropil of grey structures where tissue pathology is most severe^{24,25}, the increase of PrP^{Sc} in the target areas is conceivably higher. This evidence is even more remarkable in GSS disease, in which extensive accumulation of PrP^{Sc} in the neuropil is associated with massive deposition of PrP amyloid²⁶. Thus, in prion-related encephalopathies, neurons are exposed to high doses of amyloid protein and/or amyloid precursor protein.

The neuronal death induced by PrP106–126 occurred by apoptosis. This is regarded as a process of programmed cell death, whose activation is associated with a number of biochemical events, including selective induction of the testosterone-repressed prostate message 2 (TRPM-2) gene^{27–29}. The

FIG. 4 Apoptosis of hippocampal cells treated with PrP106–126 (a, b) and synthetic fibrils obtained *in vitro* with peptide PrP106–126 (c, d) and 127–147 (e, f). a, Photomicrograph of hippocampal cell nuclei stained with a fluorescent marker of DNA; the arrow indicates a nucleus with typical apoptotic features. b, Gel electrophoresis of hippocampal neurons DNA after 7 days of exposure to the vehicle (lanes a and b) or to 100 μ M PrP106–126 (lanes c and d). As positive control of DNA fragmentation, promyelocytic leukaemia HL-60 cells were treated with an inhibitor of protein synthesis, actinomycin D (4 μ g ml⁻¹, 5 h)¹⁷. Lanes e, f and g, h represent HL-60 control and HL-60-treated cells respectively. c, Electron micrograph of negatively stained fibrils formed by PrP106–126. Peptide assemblies consist of straight filaments with a diameter of 4–8 nm. d, Birefringence of Congo red-stained macromolecular assemblies of PrP106–126, viewed by polarization microscopy. e, Electron micrograph of negatively stained fibrils obtained with PrP127–147. Peptide assemblies consist of straight and twisted filaments 5–8 nm in diameter. f, Birefringence of Congo red-stained macromolecular assemblies of PrP127–147 viewed by polarization microscopy. Magnification: c, e, scale bar, 50 nm; d, f, scale bar, 15 μ m.

METHODS. Cell nuclei were visualized using Hoechst 33258. The cells, grown on a cover glass, were fixed in methanol/acetic acid 3:1, washed in PBS, incubated at 37 °C for 10 min with Hoechst 33258 (Calbiochem, 0.4 μ g ml⁻¹), washed in distilled water and examined under a microscope equipped for epifluorescence. To evaluate the DNA fragmentation¹⁸ the hippocampal cells from 15 mm wells were collected in PBS, pH 7.4, and kept on ice; after 15 min, lysis buffer (10 mM Tris-HCl, pH 8, 20 mM EDTA, 0.5% Triton X-100) was added, and 15 min later the solution was centrifuged (13,000g, 20 min). The supernatant was transferred to other tubes and incubated with DNase-free RNase A (100 μ g ml⁻¹); the pellets were incubated overnight with proteinase K (75 μ g ml⁻¹). DNA was extracted with phenol/chloroform solutions and the aqueous phase was precipitated in a solution of 0.5 M NaCl/isopropanol (1:10, v/v) overnight at -70 °C. DNA was resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. DNA samples were electrophoresed through 1.1% agarose gel. DNA bands were visualized by staining with ethidium bromide. DNA size markers were purchased from Boehringer-Mannheim. Assembly and aggregation properties of PrP peptides were investigated as follows. Aliquots of HPLC-purified peptides were dissolved in 5–30% formic acid at concentrations of 1, 5, 10 mg ml⁻¹, and dialysed



against distilled water at 4 °C overnight. A drop of the suspension was then applied to Formvar-coated nickel grids, negatively stained with 5% uranyl acetate and scanned with the electron microscope, or placed on gelatin-coated slides, stained with Congo red as described³⁵ and examined under polarized light.

significance of this induction is not known, but it has been suggested that TRPM-2 may be synthesized by dying cells to protect surrounding tissue³⁰. Whether apoptosis is activated *in vivo* during prion-related encephalopathies remains to be established. But note that the expression of the TRPM-2 messenger RNA is increased 10-fold in scrapie-infected hamster brains³¹.

Our data indicate that a neurotoxic mechanism is possibly responsible for neuronal cell loss in prion-related encephalopathies. This mechanism is based on chronic exposure of neurons to PrP^{Sc} and its degradation products which accumulate in brain parenchyma. Similar mechanisms could be relevant for neuronal death in other neurodegenerative disorders, including Alzheimer's disease³². □

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Correlation of expression of *Wnt-1* in developing limbs with abnormalities in growth and skeletal patterning

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THE *Wnt* genes are members of a family of vertebrate genes related to the *Drosophila* gene wingless (*wg*)^{1,2}. They encode secreted molecules^{3,4} that are thought to be important in patterning and growth control during ontogenesis^{5–7}. Several such genes are transcribed in localized domains during limb budding and morphogenesis^{2,8}. We report here a congenital limb malformation in a mouse transgenic line that ectopically expresses *Wnt-1* in the developing limbs. The hemizygote phenotype, which is inherited as an autosomal dominant trait, presents extensive distal truncations of skeletal elements, skeletal fusions and interdigital webbing. The data shown here demonstrate that abnormal *Wnt-1* expression is correlated with retarded mesenchymal condensations replaced by highly proliferative cells in the limb bud. This seems to lead to an inability of the affected cells to participate in normal skeletal development leading to the adult defects.

The function of the *Wnt-1* gene was investigated *in vivo*, in transgenic families that expressed this gene at ectopic positions. Hemizygote animals from one family showed strong skeletal malformations affecting the four limbs exclusively (Fig. 1). In the forelimbs, the radius and distal epicondyle of the humerus were absent. The bony mass was reduced distally and incomplete sets of carpals, metacarpals and phalanges were observed (Fig. 1b, c). In hindlimbs, loss of skeletal element primarily affects the autopod; digit I, its metatarsal and tarsal supporting bones were lost as well as digit II. In addition, distal phalanges were missing (Fig. 1c, h). In both fore- and hindlimbs, proximal-distal fusions were often observed. Distal truncations of tibia, fibula and ulna occurred, with fusion between fibula and talus, resulting in a remodelling of the ankle joint (Fig. 1c, f).

To determine if this dominant phenotype was linked to the ectopic expression of the *Wnt-1* gene, hemizygote transgenic

fetuses were analysed for the distribution of *Wnt-1* transcripts by *in situ* hybridization. The endogenous *Wnt-1* gene expression domain is restricted to the developing central nervous system (CNS) (Fig. 2, arrowheads) whereas this gene is not expressed in growing limbs². In transgenic embryos, however, a strong ectopic expression was detected in the limbs (Fig. 2). Ectopic *Wnt-1* messenger RNAs first appeared in day 10 (E10) fetal limbs, distally (Fig. 2a). In E11 and E12 fetuses, the transgene was highly expressed at the distal periphery of the developing limbs, in a continuous subapical domain (Fig. 2b, c). The amount of ectopic transcripts in limbs decreased by E13. Ectopic expression was also detected in the E13 dorsal thalamus (Fig. 2d, e; arrows). RNase protection analysis revealed that *Wnt-1* transcripts in limbs did not initiate at an endogenous promoter but rather in the transgene promoter, because protected fragments of the expected sizes were obtained (Fig. 2f). Because the ectopic *Wnt-1* expression domain correlated with the localizations of the phenotypic alterations (see below), we conclude that this dominant phenotype is the consequence of ectopic expression of the *Wnt-1* transgene rather than that of insertional mutagenesis, which occurs, as a dominant trait, with extreme low frequency^{9,10}.

The vertebrate limb develops in a proximal-distal sequence of growth and patterning¹¹. The growth of the structure is achieved within a distal, subectodermal area. Cells in this 'progress zone' are maintained in a highly mitotic and undifferentiated state under the influence of the apical ectodermal ridge (AER)¹² which is, in turn, stimulated by a factor(s) produced by the underlying mesenchyme cells. While the limb grows, cells continually leave this zone¹³ and condense, following a precise pattern of successive bifurcation processes¹⁴. Early condensations are composed of quiescent cells and a mantle of proliferating cells¹⁵ that overlap with the 'progress' zone at the tip of the condensations. The ectopically expressed *Wnt-1* RNA accumulates in these distal areas. The presence of the *Wnt-1* product could thus result in increased numbers of proliferating cells that do not undergo chondrogenic condensation, and could produce the observed phenotype. The analysis of hemizygote limbs at various stages of fetal development revealed a substantial enlargement of the E13 autopod along the dorsal-ventral and anterior-posterior axes (Fig. 1d, e), suggesting defective growth control.

In normal E13.5 hindlimbs, the five metatarsal blastemas are distinct and extend to the marginal zone of the footplate with

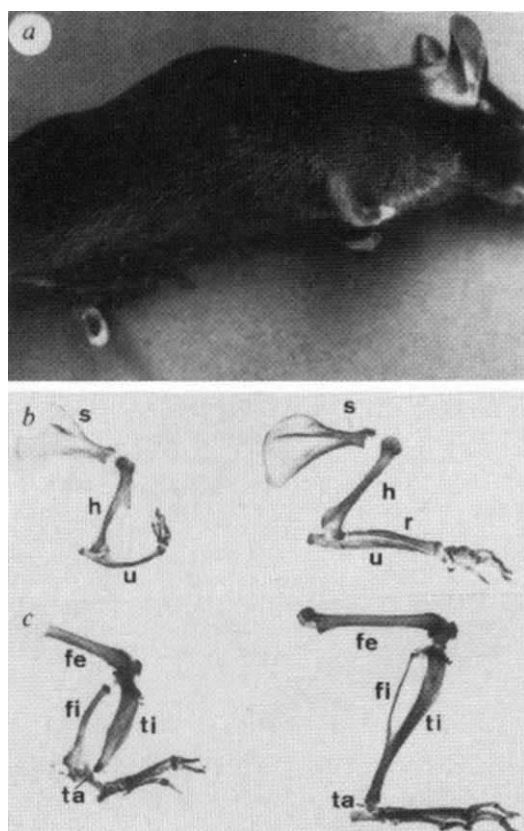
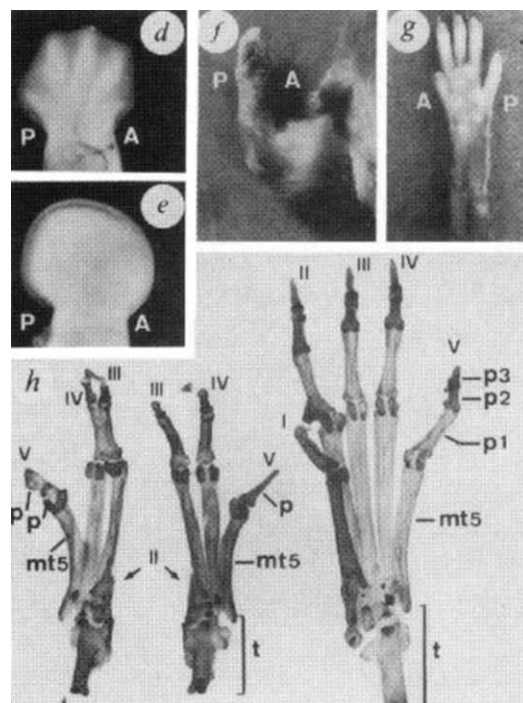


FIG. 1 Morphological characterization of the 487 transgenic family. Both male and female hemizygotes transmit the phenotype to half of their progeny. The affected animals express similar limb defects, irrespective of the sex or the parental origin of the trait. *a*, An adult male is shown to illustrate the reduction of the limbs to stumps. The paws are rotated to 90° and the overall sizes of the limbs are reduced. The mice do not show any other obvious (skeletal or otherwise) abnormality. *b*, Forelimb and *c*, hindlimb skeletons of a transgenic mouse (left) and its nontransgenic sibling (right). Most of the skeletal elements are shorter. In the forelimb (*b*), the distal half of the humerus is deficient, the radius is missing and the ulna is thinner and shorter. All the skeletons showed abnormalities in the forepaw but the extent of reductions and fusions varied from individual to individual. *c*, In hindlimbs, a severe reduction and fusions was also observed. The femur was shorter and followed by a tibia and fibula lacking their distal third. The distal end of the tibia did not articulate to the ankle and sometimes fused to the distal end of the fibula. The fibula was more voluminous proximally and fused to a deformed talus. *d*, *e*, E13 fetal hindlimbs from either normal (*d*) or transgenic fetus (*e*) are shown. Note the larger dimensions and lack of transparency of the transgenic footplate. The thickness of the abnormal limb along the dorsal-ventral axis at that stage is two- to four-fold that of control limbs. *f*, *g*, Morphologies of abnormal (*f*) and normal (*g*) littermates. Note the lack of digit separation and the reduced size of the limb as compared to the control. *h*, Two abnormal hind paws (left) are

sparser areas and indentations between the tips (Fig. 3c). These condensations are composed of elongated cells oriented perpendicular to the long axis of the rudiment that are themselves not engaged in DNA synthesis (Fig. 4c and Table 1, MC). These tightly apposed quiescent cells are surrounded by a mantle of highly proliferative mesenchyme cells (Figs 3b, 4c and Table 1, MS). In the large and opaque transgenic limbs (Fig. 1e), only the proximal halves of metatarsal condensations III and IV were apparent (Fig. 3f, g). Inside the condensations, the cells were elongated but bromodeoxyuridine (BdrU) incorporation revealed abnormally sustained DNA synthesis (Figs 3f, 4d; Table 1, MC). The other three metatarsal condensations were absent and replaced by a mass of mitotic cells (Figs 3f, 4d, arrows; Table 1, NO). Such noncondensing and growing cell



shown together with that of a normal littermate (right). In the transgenic paws, digit I as well as the corresponding metatarsal and tarsal bones are missing. The cuneiforme II and metatarsal II are very small or are absent and digit II lacks phalanges (arrows). The calcaneum articulates directly to the third cuneiforme. The third metatarsal, cuboideum, metatarsal IV and metatarsal V are present, although shorter than usual. The small plantar paired bones on the distal epiphysis of metatarsal III and IV are fused and assume different, abnormal shapes. The first phalange on III and IV are sometimes normal or fused laterally. In this latter case, they carry a single and short distal phalange. The two examples shown in *h* reveal the variability in lateral fusions of the third and fourth metatarsals and phalanges. A, anterior; P, posterior; s, scapula; h, humerus; u, ulna; r, radius; fe, femur; fi, fibula; t, tarsals; ta, talus; ti, tibia; p, phalange; mt, metatarsal.

METHODS. The injected DNA fragment was a fusion between the mouse *Hoxa-5* promoter (a 912 base pair (bp) *Bgl*II-*Sac*I fragment¹⁷) and the mouse *Wnt-1* cDNA (a 1.2 kilobase (kb) *Nco*I-*Bal*I fragment³) followed by 2.5 kb of SV40 small t-antigen 3' sequences (a *Bgl*II-*Sal*I fragment from pKSV10; Pharmacia). The predicted product of the fusion gene is the WNT-1 protein. Several lines were obtained, one of which showed limb malformations. This integration site-dependent phenotype originated from a B6D2F2 founder female. DNA preparation, microinjection, embryo transfers, animal husbandry, fetus dissections, genomic DNA isolation and Southern blotting analysis were as previously described^{18,19}. Affected animals carry one intact copy of the transgene, present at a single integration site within a 14 kb *Eco*RI genomic fragment (data not shown). The limb defect is transmitted as a dominant trait through either the females (23 out of 47 offspring) or through the males (48 out of 88). Hemizygote males do not breed well and only a few homozygotes have been obtained so far. Skeletal preparations were as described in ref. 20.

masses were detected in distal, anterior, posterior and ventral subectodermal areas, all of which expressed the ectopic *Wnt-1* transcripts. Similar investigations of earlier transgenic limbs revealed that the limb buds are already larger by day 11, in accordance with ectopic *Wnt-1* transcript accumulation in the distal subepidermal tissues first observed in E10 limbs and maintained in E11 to E13 fetuses (Figs 2b, c and 3e). Later, when phalangeal condensations are normally being completed (E14), the limbs already exhibited significant size reduction, absence of fingers and an indented zeugopod, reflecting the adult defects.

In summary, ectopic *Wnt-1*-specific RNA accumulation first predates (Fig. 2b, c) and then colocalizes with the accumulation of noncondensing, proliferating mesenchymal cells.

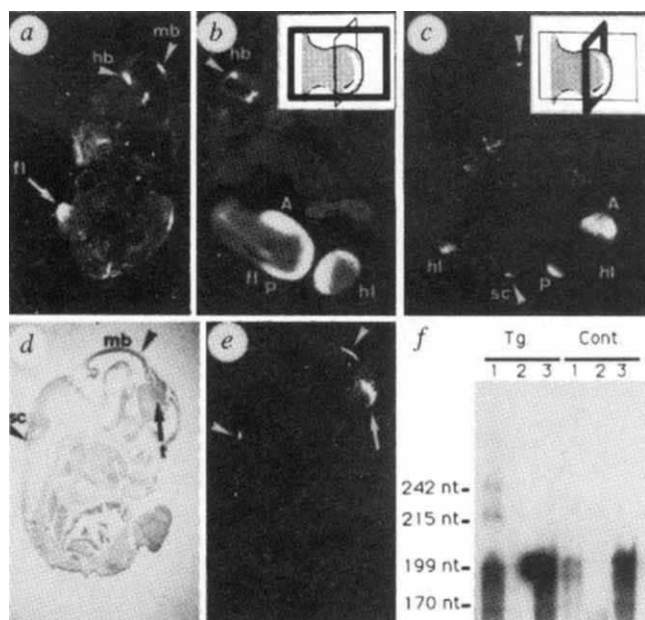


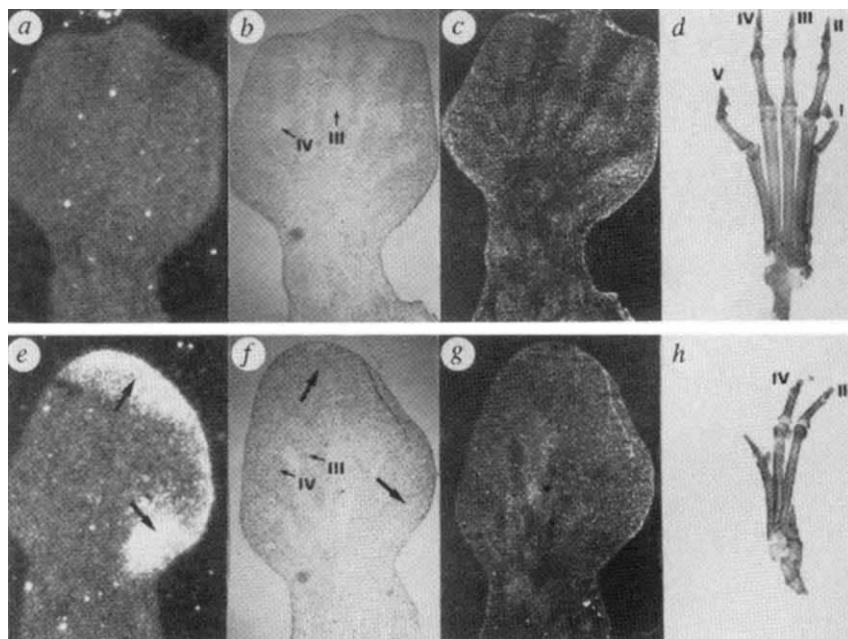
FIG. 2 Detection of *Wnt-1*-specific RNA in transgenic E10, 11.5, 12 and 13.5 fetuses by *in situ* hybridization and RNase protection. *a*, Parasagittal

FIG. 3 Comparative analysis of 13-day-old normal (*a-d*) and transgenic (*e-h*) hindlimbs. *a, e*, The sections were hybridized *in situ* with the *Wnt-1*-specific probe. Although the normal limb is negative, the transgenic limb expresses detectable levels of *Wnt-1* transcripts, most concentrated at the distal and anterior periphery of the limb (*e*, arrows). Neighbour histological sections are shown in bright-field illumination (*b, f*) or with phase-contrast microscopy (*c, g*). The dark grains in *b* and *f* show the distribution of nuclei engaged in DNA synthesis as revealed by immunoperoxidase detection of BdrU incorporated *in vivo*. Note the alignment of staining with the periphery of the condensations in contrast to the lack of staining inside the condensed precartilagenous mesenchyme. In the transgenic limb, the distribution of positive nuclei is severely perturbed. In distal and preaxial regions, the distribution of labelling is homogeneous. In addition, the level of signal is higher than normal inside condensations III and IV where the precartilagenous mesenchyme still shows a high BdrU incorporation. The examination of the same sections by phase contrast confirmed that the loss of distal unlabelled areas in *f* coincides with the absence of condensed material in *g*. Thus, the absence of the first and second metatarsal condensations correlates with the presence of a homogeneous BdrU labelled cellular mass in the abnormal specimen (arrows in *f, g*). The condensations in the normal limb extend to the marginal area (*c*) whereas the condensations III, IV and V of the abnormal limb are shorter (*g*). The locations of these masses of noncondensed cells correlate well with sites at which the *Wnt-1* gene is ectopically transcribed (compare arrows in *e* with *f* and *g*). The skeletal patterns obtained in both case are shown in *d* and *h*. Note the general deficit in bones in regions that correspond to the *Wnt-1* expression domains, the presence of labelled nuclei and to the absence of mesenchymal

Condensations are observed in more proximal or central parts of the limbs, where *Wnt-1* expression is either weaker or not seen (Figs 2*b, c* and 3*e-g*). This spatial and temporal correlation suggests a direct effect of the WNT-1 protein on the retardation of mesenchymal condensation and the accumulation of proliferating mesenchymal cells. These alterations in limb development may result from an interference between the ectopic WNT-1

sections of a day 10 transgenic embryo. The section was hybridized with a *Wnt-1*-specific antisense RNA probe. Arrowheads indicate the endogenous signals in the midbrain (mb), hindbrain (hb) and spinal cord (sc; see ref. 21). A specific ectopic signal is seen in the forelimb bud (fb; arrow). In parasagittal and transverse sections of 11.5- and 12-day-old specimen (*b* and *c*, respectively), a strong signal is detected in the most distal and peripheral parts of both forelimbs (fb) and hindlimbs (hl). The planes of section through the limbs shown in *b* and *c* are depicted by the bold lines in the corresponding schemes in the upper right corners. These planes are orthogonal to one another. *Wnt-1* endogenous expression domains are shown with arrowheads. *d, e*, Bright-field (*d*) and dark-field (*e*) views of a skewed sagittal section of a 13.5-day-old transgenic fetus hybridized with the same *Wnt-1* probe. The endogenous *Wnt-1* signal is detected in the roofplate of the midbrain (mb) and spinal cord (sc). Ectopic signal is seen in the dorsal thalamus (t). *f*, *Wnt-1* RNAse protection analysis using 12.5-day-old fetal RNA from either transgenic (Tg) or normal (Cont.) embryos. The RNA is from: 1, limbs and associated trunk tissue; 2, midbrain; 3, remainder of the embryonic tissues. The 199 nucleotide (nt) protected fragments in samples 1 and 3 correspond to *Hoxa-5* endogenous transcripts¹⁷. The 215- and 242-nt-long protected fragments (present in Tg lane 1 but absent from Cont. lane 1) correspond to the expected fusion transcripts that would initiate at previously mapped transcription start sites¹⁷.

METHODS. Tissue fixation, histological processing and *in situ* hybridization were done as described in ref. 22. The *Wnt-1* antisense RNA used as the hybridization probe was produced from the *NcoI*-*BalI* cDNA fragment derived from clone S621 (ref. 3). Transgenic fetuses were entirely sectioned and series of neighbour sections were examined. For the RNAse protection analysis, 30 µg of total RNA was hybridized to a 415 bp antisense transcript (an *XbaI*-*XhoI*, *Hoxa-5/Wnt-1* fusion fragment) according to established procedures.



condensations.

METHODS. A pregnant female was injected on the 13th day of pregnancy with 50 mg kg⁻¹ of BdrU in distilled water. One hour later, the fetuses were fixed in 4% paraformaldehyde overnight and stored at 4 °C in 20% sucrose. Transgenic and normal limbs were dissected out and embedded in the same block. Cryostat sections, immunohistochemical detection of BdrU and *in situ* hybridization detection of *Wnt-1* specific RNA were done according to standard procedures^{22,23}.

protein and other members of the family of WNT proteins which are normally present⁸, as has been suggested for mammary gland tumorigenesis¹⁶. For example, the ectopically produced protein could, through endogenous WNT receptor molecules, prevent distal cells from condensing by maintaining them in a large, abnormal 'progress zone'.

The deletions of skeletal elements observed in the adults reflect

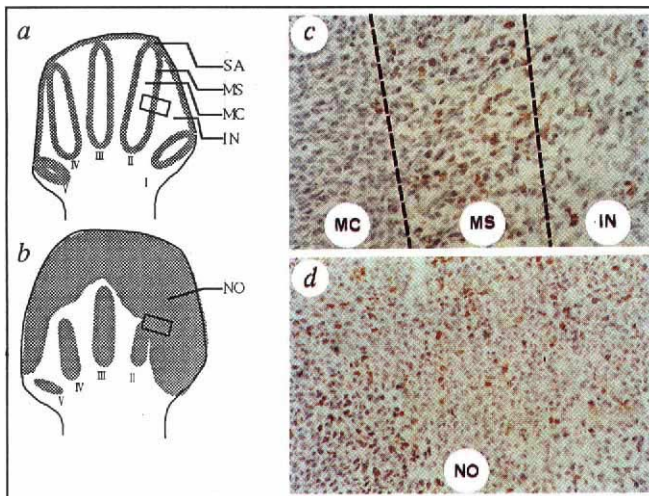


FIG. 4 Distribution of proliferating mesenchyme cells in fetal autopods. *a*, *b*, Schematic representations of either normal (*a*) or transgenic (*b*) E13 autopods. Shaded areas represent regions with a high occurrence of cells that incorporated BdrU. *c*, *d*, Distribution of BdrU-positive (brown) cells in comparable areas (rectangles in *a* and *b*) from normal and transgenic limbs, respectively. In the normal autopod (*c*), such cells are abundantly present at the surface of the metatarsal condensations whereas the core of the condensation is devoid of them. In contrast, cells in S phase are found scattered throughout *d*. The various areas indicated in *a* and *b* are those which were used for counting and which are included in Table 1. They are: the subapical area (SA), the periphery of the metatarsal (MS), the centre of the metatarsal (MC), the intermetatarsal space (IN) and, in transgenic footplate, the noncondensed material (NO). The dashed lines in *c* shows the transitions between different areas.

the inability of these proliferating cells to condense and undergo chondrogenesis. This leads to the observed paradoxical situation of larger fetal limbs, because of more progress-zone-like cells, but truncated adult limbs resulting from the transitory block in chondrogenesis. This interpretation further suggests that members of the *Wnt* gene family may be mediating epithelial-mesenchymal interactions that control limb growth and may thus act at the interface between growth and patterning. □

TABLE 1 Distribution of cell densities and DNA synthetic activity in control and transgenic 13.5-day-old hindlimbs

Selected areas* in the autopod	Control Cell no. (s.e.)/(%)	Transgenic Cell no. (s.e.)/(%)
Subapical (SA)	173.3 (9.7)/(18)	197.0 (6.2)/(18)
Periphery of metatarsal (MS)	116.0 (4.9)/(28)	132.0 (7.2)/(28)
Centre of metatarsal (MC)	134.7 (8.9)/(1)	171.6 (6.7)/(18)
Intermetatarsal space (IN)	84.0 (2.4)/(8)	92.3 (8.2)/(6)
Noncondensed material (NO)‡	—	120.7 (4.9)/(26)

Cell densities given as number of cells per arbitrary surface unit (mean values). DNA synthetic activity expressed in percentages of BdrU-positive cells.

* The various selected areas are depicted in Fig. 4*a*, *b*.

† Standard error.

‡ Other than SA, MS or IN, present only in transgenic limbs.

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Interaction between a transcriptional activator and transcription factor IIB *in vivo*

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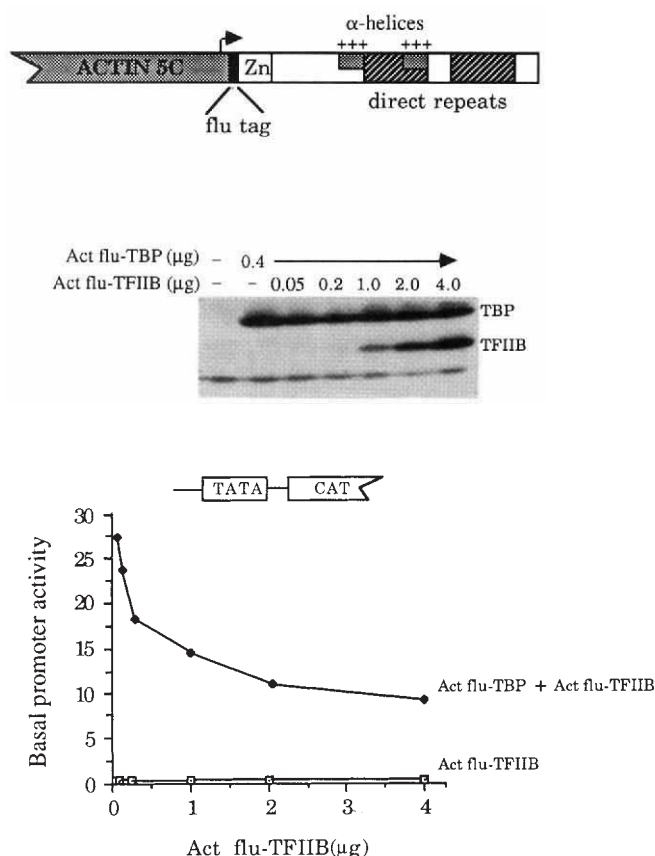
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TRANSCRIPTION of messenger RNA-encoding genes *in vitro* requires many protein factors^{1,2}. Transcription factor IID, possibly with the cooperation of TFIIA, binds to the TATA element of the promoter^{3–5}, forming a complex that can bind TFIIB (refs 6, 7) followed by RNA polymerase II (refs 6, 8) and other factors⁹. One or more of these steps is thought to be facilitated by gene-specific transcriptional activation proteins^{10–12}; this seems to require TFIID-associated auxiliary factors^{13–15} and may involve direct contact between the activator and TFIID^{16,17} and/or TFIIB^{18,19}. If such contact is necessary *in vivo*, activation might conceivably be blocked by a TFIIB derivative containing the sequences necessary for this interaction, but lacking those necessary for

binding to the rest of the transcriptional apparatus, an effect similar to that referred to as squelching²⁰ or transcriptional interference²¹. Here we show that the activity of the glutamine-rich *fushi tarazu* activation domain is indeed blocked by truncated TFIIB derivatives in *Drosophila* Schneider L2 cells, suggesting that it is mediated by interactions with TFIIB.

An expression plasmid (Act flu-TFIIB; Fig. 1) encoding the 315-amino-acid *Drosophila* TFIIB protein^{22,23} fused at the amino terminus to an 11-amino-acid epitope tag^{24,25}, was transfected into *Drosophila* Schneider L2 cells. Western blot analysis confirmed that the protein was expressed in a concentration-dependent fashion in the transfected cells (Fig. 1).

To test whether overexpressing TFIIB affected expression from a minimal TATA-containing promoter, L2 cells were cotransfected with increasing concentrations of Act 'flu-TFIIB and a reporter plasmid (E1b TATA CAT) containing only the adenovirus E1b TATA element upstream of the chloramphenicol acetyl transferase (CAT) gene. No increase in CAT expression was detected, indicating that TFIIB is not limiting for basal promoter activity (Fig. 1). Co-expression of TFIIB and the TATA-binding subunit of TFIID (TBP), whose overexpression can increase promoter activity²⁶, produces slight concentration-dependent reductions (2–3-fold) in TBP-enhanced promoter activity (Fig. 1), suggesting that increases in the intracellular TFIIB concentration cannot bring about a more



productive association with TFIID. Increased synthesis of TFIIB had little effect at any level of target promoter expression, demonstrating that TFIIB is not limiting for promoter activity within transfected cells.

To test the effects of TFIIB derivatives on transcription activation, a series of expression vectors encoding N- or C-terminally truncated forms of TFIIB fused to the influenza epitope were assayed for their ability to affect expression either from E1b TATA CAT stimulated by TBP overexpression²⁶, or from an otherwise identical reporter plasmid containing GAL4 binding sites (G5 E1b TATA CAT) activated to a similar level by expression of the chimaeric activator protein GAL4-*ftzQ* (Fig. 2a). N-terminally truncated TFIIB derivatives caused only minor inhibition of enhanced basal expression and had essentially no effect on activation by GAL4-*ftzQ*. C-terminally truncated TFIIB derivatives only slightly reduced TBP-enhanced expression but significantly inhibited activation by GAL4-*ftzQ*. Reductions of up to ~75-fold were observed with a derivative lacking 113 C-terminal residues (flu-TFIIBΔC202), despite the reduced accumulation of all three of the C-terminal truncations. This inhibition was not due to changes in the steady-state level of GAL4-*ftzQ* protein (Fig. 2a). The low basal promoter activity detected in the absence of GAL4-*ftzQ* was only slightly reduced by flu-TFIIBΔC202, showing that the inhibition is specific to activated transcription.

Flu-TFIIBΔC202 contains two regions (amino acids 104–126 and 184–201) that are capable of forming amphipathic α-helices (ref. 22; Fig. 2b) and a region (amino acids 14–35) that can form a zinc-finger^{22,23,27}. To determine whether these or other sequences are required to inhibit activation by GAL4-*ftzQ*, derivatives of flu-TFIIBΔC202 lacking stretches of 13 to 37 amino acids were tested (Fig. 2b). Mutants lacking stretches between residue 48 and the C terminus of flu-TFIIBΔC202 all inhibited activation, although two mutants (flu-TFIIBΔC170 and Δ121–157ΔC202) were somewhat less effective, perhaps reflecting their reduced accumulation. By contrast, the double

FIG. 1 Effects of TFIIB overexpression on CAT gene expression from a minimal TATA-containing promoter *in vivo*. Schematic at the top depicts the structure of the 'flu epitope-Drosophila TFIIB fusion protein expression vector (Act flu-TFIIB). Specific structural features of TFIIB have been described^{7,22,23,27}. Western blots show the accumulation of flu-TFIIB fusion proteins in cells transfected with the indicated concentrations of Act flu-TFIIB, and a constant amount (0.4 μg) of a *Drosophila* flu-TBP expression plasmid (Act flu-TBP)²⁶ as indicated. Graph shows the effects of increasing concentrations of Act flu-TFIIB, either alone or plus a constant amount (0.4 μg) of Act flu-TBP, on expression from a reporter gene consisting of the adenovirus E1b TATA element upstream of the CAT gene, which is represented schematically above the graph. Promoter activity is shown in βG units (Fig. 2a legend).

METHODS. Act flu-TFIIB was constructed using a bacterial expression vector encoding the *Drosophila* TFIIB²³ and two previously described *Drosophila* expression plasmids (Act-PPA, pActPfl)^{26,29}. Act flu-TBP, formerly referred to as Act flu-TFIID, has been described²⁶. The reporter construct used is a derivative of the plasmid E1b TATA CAT (ref. 26). Transfections were done essentially as described²⁹. The amount of expression vector added to all transfection mixtures was kept constant by addition of expression vector (Act-PPA) lacking any cDNA insert as needed. Each transfection was performed independently at least 4 times, usually twice in duplicate. Western blot analysis was as described²⁶, except the secondary antibody used was an unconjugated rabbit anti-mouse IgG, which was detected using an ¹²⁵I-labelled goat anti-rabbit IgG antibody.

mutant flu-TFIIBΔN23ΔC202, which lacks the N-terminal residues necessary to form the potential zinc-finger, did not inhibit activation by GAL4-*ftzQ*, indicating that the N-terminal sequences of TFIIB are necessary for inhibition.

Flu-TFIIBΔC202 thus seems to interact with GAL4-*ftzQ* without functioning in transcription complex assembly, thereby blocking the effect of the activator. The TFIIB mutant might not interact with the co-expressed activator directly, however, but might instead titrate a required co-activator, for example. If inhibition does involve a direct interaction between flu-TFIIBΔC202 and GAL4-*ftzQ*, it should be overcome by increasing the concentration of the activator relative to the inhibitor. The effect of increasing concentrations of Act GAL4-*ftzQ* on inhibition caused by a constant amount of Act flu-TFIIBΔC202 was therefore determined (Fig. 3a). Inhibition decreased from 105 to 6-fold as the level of GAL4-*ftzQ* expression vector was increased, implying that inhibition of GAL4-*ftzQ*-mediated activation involves direct interaction between this factor and flu-TFIIBΔC202. If the inhibition observed reflects a physiologically relevant interaction between TFIIB and Gal4-*ftzQ*, rather than an artefact such as misfolding of the mutant TFIIB, it should be possible to reverse it by increasing the concentration of wild-type TFIIB (Fig. 3b). Cotransfection of Act flu-TFIIB (which by itself did not affect activated or basal expression; see Fig. 1) and Act flu-TFIIBΔC202 fully restored activation by GAL4-*ftzQ*, unlike cotransfection of Act flu-TFIIBΔC202 and Act flu-TFIIBΔN23. This was not due to differential accumulation of the two proteins, as flu-TFIIBΔN23 and flu-TFIIB were detected in similar amounts (data not shown). The N terminus of native TFIIB thus seems to interact with GAL4-*ftzQ*.

Our results suggest that a direct, functional interaction between TFIIB and an activator (GAL4-*ftzQ*) can regulate transcription *in vivo*, and a simple model consistent with our data is presented in Fig. 4. In the absence of activator, TFIID and TFIIB can interact, resulting in low promoter activity limited by the concentration of available TFIID (Fig. 4a). In the

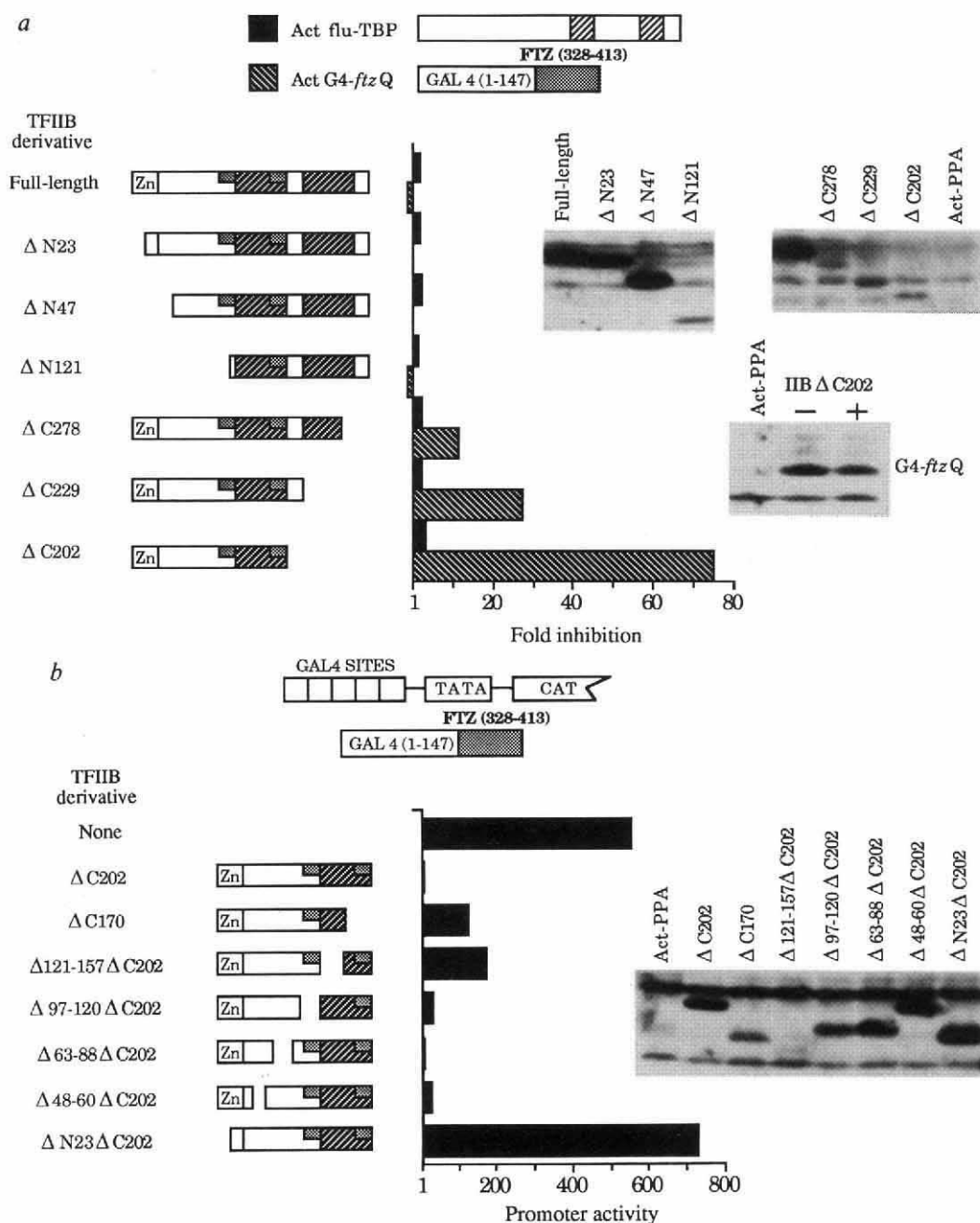


FIG. 2 Effects of truncated forms of TFIIIB on activation mediated by TBP or GAL4-ftzQ. **a**, Bar graph showing the effects of coexpressing N- and C-terminally truncated forms of TFIIIB, which are depicted schematically on the left of the graph, on activation by TBP or GAL4-ftzQ, which are represented schematically at the top. Hatched boxes represent two direct repeats found in the C terminus of TBP. GAL4-ftzQ consists of the DNA-binding domain of the yeast activator GAL4²⁸ fused to an 85-amino-acid glutamine-rich activation domain naturally found at the C terminus of the *Drosophila* homeodomain protein Fushi tarazu^{29,30}. Reporter plasmid activities were determined in β G units, which represent the ratio between CAT activity and β -galactosidase activity expressed from the internal control plasmid copia-lacZ²⁹. (Expression from copia-lacZ was unaffected by any of the expression vectors used in this study.) Activated expression in β G units was 250 for TBP and 310 for GAL4-ftzQ. Basal expression from G5 E1b TATA CAT measured 5.6 β G units and was reduced to 1.1 by flu-TFIIIB Δ C202. Background measured 0.17 β G units, as determined from transfections containing an otherwise identical reporter plasmid lacking the E1b TATA element. Western blots at the top right show the level of accumulation of the indicated flu-TFIIIB fusion protein. Western blot at the lower right shows the accumulation of the GAL4-ftzQ protein in the presence or absence of the Act flu-TFIIIB Δ C202 expression plasmid. Accumulation of flu-TFIIIB Δ C202 was not affected by addition of Act GAL4-ftzQ (data not shown). Lanes marked Act-PPA contain extract from cells transfected with the expression vector

(Act-PPA) lacking a cDNA insert. **b**, Bar graph showing the effects of expressing the indicated mutant forms of TFIIIB on activation by GAL4-ftzQ. Promoter activity is shown in β G units. Mutant TFIIIB proteins are represented schematically on the left of the graph, and the GAL4-ftzQ activator protein and G5 E1b TATA CAT reporter gene are depicted schematically at the top. Western blot on the right shows the level of accumulation of each flu-TFIIIB protein.

METHODS. TFIIIB mutant expression plasmids were constructed from Act flu-TFIIIB using standard procedures. The Act GAL4-ftzQ expression construct was created using the plasmids pAct5Cftz²⁹ and pSG424 (ref. 31). G5 E1b TATA CAT contains 5 synthetic GAL4 binding sites inserted upstream of the E1b TATA element. The bar graph in **a** displays data from transfections containing either 2.4 μ g Act TBP (ref. 26), or 3.0 ng Act GAL4-ftzQ, with or without 2.0 μ g of the appropriate TFIIIB expression plasmid. The bar graph in **b** shows data from transfections containing 7.0 ng Act GAL4-ftzQ, with or without 4.0 μ g of the indicated TFIIIB expression plasmid. Western blot analysis was done as described for Fig. 1 on extracts from cells transfected with 4.0 μ g of the indicated TFIIIB expression plasmid. To detect GAL4-ftzQ expression, extracts from cells transfected with 0.2 μ g Act GAL4-ftzQ plus or minus 2.0 μ g Act flu-TFIIIB Δ C202 were probed with antisera raised against the first 147 amino acids of GAL4, followed by incubation with an [¹²⁵I]-labelled goat anti-rabbit IgG antibody. The amount of expression vector added was kept constant by addition of Act-PPA.

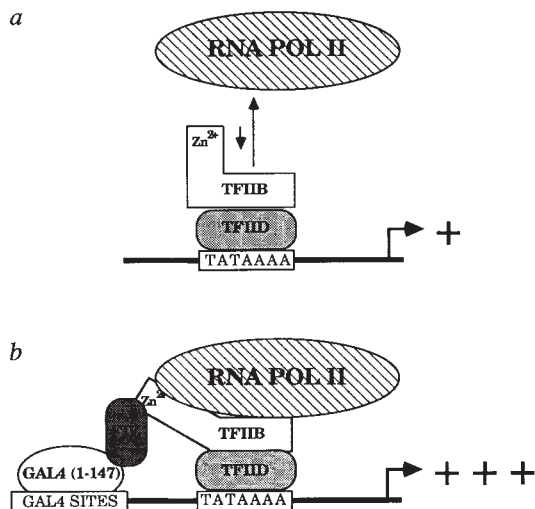
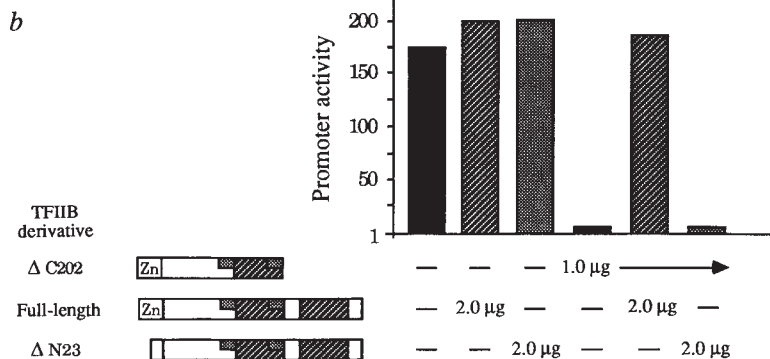
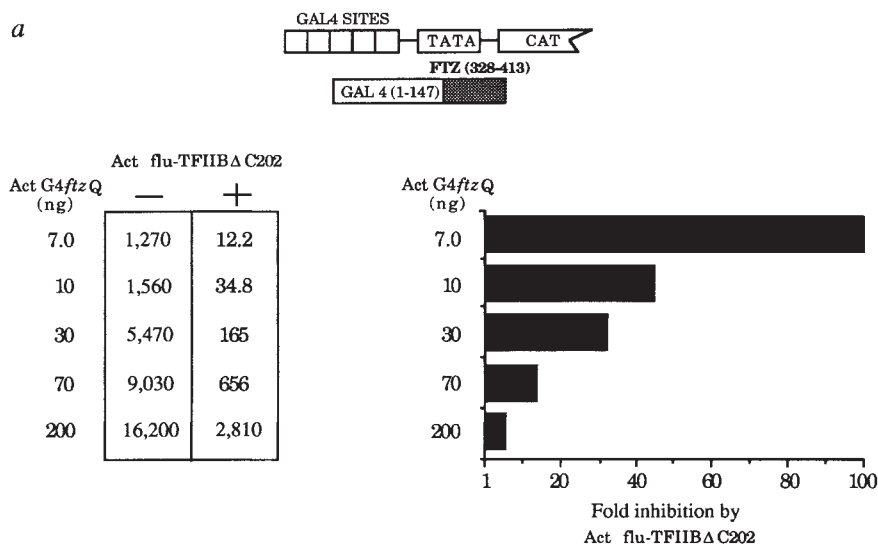


FIG. 4. Model explaining the effects of interactions between TFIIB and GAL4-*ftzQ*. For simplicity, basal factors other than TFIID and B are not shown, nor are subunits of the TFIID complex indicated. *a*, TFIID, TFIIB and RNA polymerase can associate to some degree *in vivo*, which can be facilitated by overexpression of the TATA-binding subunit of TFIID, resulting in moderate promoter activity. *b*, Interactions between GAL4-*ftzQ* and TFIIB cause a conformational change in TFIIB which allows it to interact more stably with TFIID and/or RNA polymerase II, which consequently increases promoter activity.

FIG. 3 Effects of increasing concentrations of GAL4-*ftzQ* or full-length TFIIIB on inhibition by flu-TFIIIB Δ C202. *a*, Table shows promoter activity (in β G units; Fig. 2*a* legend) obtained by adding increasing concentrations of the Act GAL4-*ftzQ* (7.0, 30, 70 or 200 ng) expression plasmid plus or minus a constant amount (2.0 μ g) of the Act flu-TFIIIB Δ C202 expression plasmid. The bar graph shows the effects of adding the indicated concentrations of Act GAL4-*ftzQ* on inhibition mediated by 2.0 μ g Act flu-TFIIIB Δ C202. The activator protein and reporter plasmid used are depicted schematically at the top. *b*, Bar graph displaying the effects of adding the indicated combinations of Act flu-TFIIIB, Act flu-TFIIIB Δ N23 and Act flu-TFIIIB Δ C202, on activation by Gal4-*ftzQ*. Promoter activity is shown in β G units. Activator protein and CAT reporter plasmid used are depicted schematically at the top in *a*. TFIIIB expression vectors used are represented schematically at the bottom left of the bar graph, and the amounts included of each are indicated below the appropriate bar.

METHODS. All expression and reporter plasmids are described in the legends to Figs 1 and 2. Transfections were as described for Fig. 1. Bar graph in *b* shows data from transfections containing 3.0 ng Act Gal4-*ftzQ* with the indicated amount of each TFIIIB expression plasmid. The amount of expression vector added was kept constant by addition of Act-PPA.

presence of GAL4-*ftzQ*, contact between the activator and the N-terminal region of TFIIB causes much higher levels of promoter activity, perhaps by inducing a conformational change that allows TFIIB to interact more stably with TFIID and/or some other component of the transcriptional apparatus, such as RNA polymerase II (Fig. 4*b*). Other activators, such as GAL4-VP16, may also interact directly with TFIIB^{18,19}, but experiments analogous to those described here using GAL4-VP16 as the activator showed only slight inhibition by the TFIIB mutants (unpublished data). VP16 may thus interact with a different region of TFIIB, with TFIID-associated factors (TAFs), and/or with TBP. Although our findings do not rule out interactions between GAL4-*ftzQ* and other proteins, they nonetheless indicate that TFIIB is a target for a transcriptional activator *in vivo*. □

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Human lysozyme gene mutations cause hereditary systemic amyloidosis

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HEREDITARY non-neuropathic systemic amyloidosis (Ostertag-type)¹ is a rare autosomal dominant disease in which amyloid deposition in the viscera is usually fatal by the fifth decade. In some families it is caused by mutations in the apolipoprotein AI gene^{2,3} but in two unrelated English families under our care the amyloid deposits did not contain apoAI, despite a report that this may have been the case in one of them⁴. Lysozyme is a ubiquitous bacteriolytic enzyme present in external secretions⁵ and in polymorphs and macrophages, but its physiological role is not always clear⁶. Here we report that in these two families, lysozyme is the amyloid fibril protein. Affected individuals are heterozygous for point mutations in the lysozyme gene that cause substitution of highly conserved residues, namely threonine for isoleucine at position 56 in one family, and histidine for aspartic acid at residue 67 in the other. Amyloid fibrils from one individual were composed of the full-length Thr-56 variant lysozyme molecule. To our knowledge, this is the first report of naturally occurring variants of human lysozyme and of lysozyme-associated disease. As the structures of human⁷ and hen egg-white lysozyme⁸ are known to atomic resolution and their folding and structure-function relationships have been exhaustively analysed, our observations should provide a powerful model for understanding amyloidogenesis.

Amyloid fibrils extracted from the only unfixed tissue of the last surviving affected individual in one family⁴ (family 1, Fig. 1) yielded a single major protein which ran at a position corresponding to an M_r of 15,000 (15K) in SDS-PAGE, had the same N-terminal sequence as mature human lysozyme, and reacted with antibodies to lysozyme (Figs 2, 3). Immunohistochemical staining of all available amyloidotic tissues from the affected members of both families (Fig. 1) confirmed that the deposits consisted of lysozyme (Fig. 2). A subcutaneous fat biopsy from one of the living patients in family 2, who had massive systemic amyloidosis ('H' in Fig. 1), contained insufficient amyloid for fibrils to be extracted, but lysozyme was detected immunohistochemically (Fig. 2). In contrast, amyloidotic tissues from patients with acquired and hereditary amyloidosis (transthyretin and apoAI variants) did not stain with anti-lysozyme antibodies, nor did senile amyloid of articular cartilage⁹ or localized ocular amyloid deposits¹⁰, which we tested because of the high concentration of lysozyme in articular cartilage and in tears⁶.

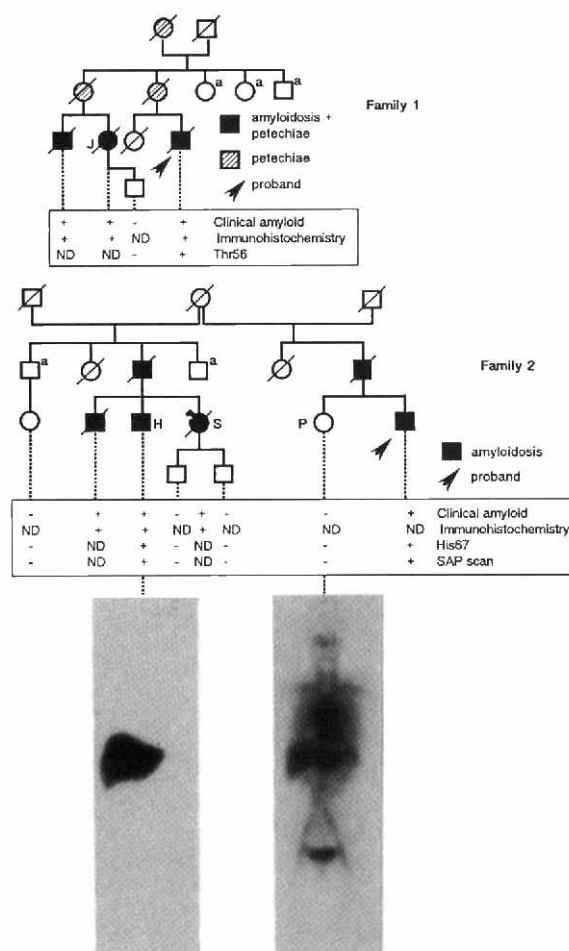


FIG. 1 Family trees of two kindreds with hereditary systemic non-neuropathic amyloidosis. In family 1 all members with amyloidosis had a petechial skin rash from childhood, as did some members of previous generations⁴. The presence of amyloid in the two earlier generations is not known, although the proband's mother died of renal failure, compatible with amyloidosis⁴. The proband's sister died aged 8 in a motor accident. In family 2 the proband is the individual previously reported²⁰. Shown below the tree of family 2 are anterior whole-body scintigraphic images of two family members 24 h after intravenous injection of ¹²⁵I-labelled human serum amyloid P component (SAP). This procedure is a sensitive and specific quantitative *in vivo* diagnostic test for amyloid deposits²¹. In the healthy relative P, the tracer is seen only in the bloodstream with no tissue deposition or localization; radioactive breakdown products excreted in the urine give an image of the bladder. In the amyloid patient H, the tracer has localized to the massive amyloid deposits in the enlarged liver. The liver signal is so strong that localization to amyloid elsewhere cannot be visualized. There is no blood pool signal nor any breakdown products in the urine, indicating that the patient has a very heavy whole-body amyloid load. The legends to the family trees are as follows. Clinical amyloid: symptomatic systemic amyloidosis confirmed histologically on biopsy or autopsy specimens by staining with Congo red²²; immunohistochemistry: immunohistochemical staining of amyloid deposits with anti-lysozyme antibodies (Fig. 2); Thr 56 or His 67: lysozyme variants identified by gene sequencing (Fig. 3); SAP scan: *in vivo* scintigraphic imaging of amyloid deposits with ¹²⁵I-labelled SAP; ND: not done because the patient was dead and/or tissue was not available; a: present whereabouts not known; a line through a symbol indicates that that person is deceased.

We then sequenced the lysozyme gene in all members of these families from whom DNA was available. The proband in family 1 was heterozygous for a T→C transition in exon 2, altering codon 56 from Ile to Thr (Fig. 3). The one available surviving unaffected relative (the son of J.; Fig. 1) was homozygous for the wild-type sequence. In family 2 both surviving affected members were heterozygous for a G→C transversion in exon 2 of the lysozyme gene, encoding a His for Asp substitution at residue 67 (Fig. 3). Four other family members who did not have amyloid were homozygous for the wild-type sequence in exon 2, and the remainder of the coding sequence was normal in all cases.

Sequencing of cyanogen bromide fragments of the amyloid fibrils from the proband in family 1 demonstrated only the presence of Thr, the variant residue, at position 56 (Fig. 3). Laser desorption mass analysis (Finnegan LASERMAT) of the fibrils revealed a major component mass of 14.680K, corresponding to the calculated mass for the whole variant lysozyme molecule including the Thr-56 substitution. Although the precision of this method is not sufficient to rely on the result for identification of the residue 56 substitution, it confirms that the fibril subunits are whole, intact lysozyme molecules. The fibrils were solubilized *in vitro* only by 90% formic acid or 6 M guanidine hydrochloride, and are thus much less soluble than normal lysozyme. They were also highly resistant to degradation by proteinases (see legend to Fig. 3).

No natural mutations in the human lysozyme gene, nor any variation in amino-acid sequence have been reported previously.

Concordance of the lysozyme gene mutations with amyloidosis in both families, coupled with demonstration of the variant lysozyme protein as amyloid fibrils, establish that these mutations cause the disease.

The fully refined structure of human lysozyme at 1.5 Å resolution⁷ was used to evaluate the possible effects of the variant residues. Residue 67 (66 in hen¹¹ and some other lysozymes) is an invariant Asp in all listed lysozyme sequences¹². It has an abnormally low pKa¹³, which is accounted for by its role in stabilizing the external 64–76 loop in which the carboxylate group of the side chain acts as a hydrogen-bond acceptor to the hydroxyl group of Tyr 54, the main-chain –NH of residue 69, and the –OH group of Thr 70 (Fig. 4a). This stabilizing role cannot be played by histidine, which is too large to fit into the centre of the loop and cannot accept three hydrogen bonds. As even in the presence of aspartic acid the loop has the highest B-factors in the human lysozyme molecule¹⁴, loss of the stabilizing factor is likely to result in disruption of the structure of the loop. Alteration of the loop structure could generate the site that accounts for aggregation into amyloid fibrils, and interestingly it is closely associated to the β-sheet region of the molecule, a motif frequently linked with amyloid formation.

Residue 56 is aliphatic, being Ile (as in human lysozyme), Leu or Val in all known lysozyme sequences¹². It is located in a highly conserved region at the base of the β-sheet, with the side chain buried in the protein core (Fig. 4b). The isostructural Val in the normal sequence of Californian quail lysozyme, and the observation that there is 'little structural change' when Val

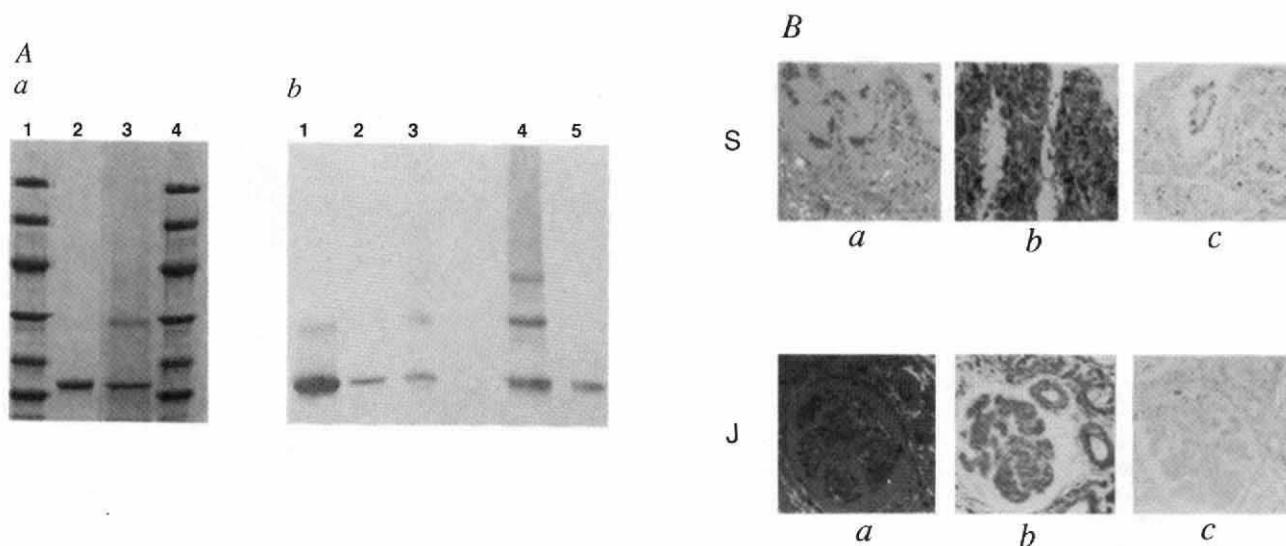


FIG. 2 A, a, SDS 8–18% gradient PAGE (Pharmacia) analysis of isolated amyloid fibrils from the proband in family (Fig. 1). Fibrils were extracted²³ from an unfixed fragment of amyloid-laden renal tissue obtained at autopsy in 1984 and stored frozen at -80°C until 1992. A reduced denatured 10 μg sample of the fibrils was run in lane 3; lane 2 contains 2.5 μg human breast-milk lysozyme (Sigma); lanes 1 and 4 contain marker proteins (97K, 67K, 43K, 30K, 21.1K, 14.4K). The gel was stained with Brilliant blue R350. B, Immunoblot stained with rabbit anti-lysozyme antibodies (Dako) and peroxidase-labelled goat anti-rabbit IgG (Dako). Lanes 1 and 2, human breast-milk lysozyme (Sigma), 2.5 μg and 0.25 μg ; lane 3, subcutaneous fat from patient H, family 2 (Fig. 1); lane 4, isolated amyloid fibrils (10 μg) from proband, family 1 (Fig. 1); lane 5, normal human saliva. The fresh unfixed fat biopsy was washed in saline, delipidated, dissolved in 6 M guanidine hydrochloride, dialysed against water and lyophilized. A 2-mm³ fragment was then dissolved in 100 μl SDS sample buffer by heating at 100°C for 5 min. Normal saliva used as a source of lysozyme for comparison was diluted 1:2 with SDS sample buffer and boiled. Reduced denatured samples (10 μl) were run in SDS 8–18% gradient PAGE (Pharmacia) and then transferred to Problott 0.2- μm PVDF membrane (Applied Biosystems) in the

Pharmacia Novablot semidry blotting apparatus with continuous buffer system. The membrane was immunostained using standard procedures (Problott) and photographed before drying. Lanes 1 and 3 show traces of dimers and oligomers as frequently seen in preparations of isolated human lysozyme. Subcutaneous fat removed during routine surgery on normal control subjects did not yield material that reacted with antibodies against lysozyme (not shown). B, Histochemical staining of amyloid deposits in formalin-fixed tissues from post-mortem examinations of patients J (family 1) and S (family 2) (Fig. 1); a, Congo red stain²² viewed in polarized light showed the pathognomonic green birefringence of amyloid, seen here as bright areas; b, indirect immunoperoxidase stain (PAP method²⁴) using rabbit antiserum to human lysozyme (Dako), showing abundant dark areas corresponding to the peroxidase reaction product on amyloid deposits; c, antigen absorption specificity control for anti-lysozyme staining in which the antiserum was absorbed with an excess of pure human lysozyme (Sigma) before use, and showing complete absence of peroxidase reaction product on the amyloid. The colonic mucosa of patient J was packed with amyloid deposits, as were the glomeruli, blood vessel walls, tubules and interstitium of the renal cortex in patient S. Magnification, $\times 98$.

is introduced by site-directed mutagenesis at the position equivalent to 56 in hen egg-white lysozyme¹⁵, indicate that the hydroxyl group of the variant Thr is the key change. Difficulty in burying this polar hydroxyl group in the hydrophobic core could alter folding of the variant lysozyme, thus exposing an amyloidogenic determinant. If, however, the protein folds normally, the hydroxyl group of the buried Thr 56 could hydrogen-bond to the hydroxyl of Ser 36, a normally buried residue

(Fig. 4c). This possibility was explored using the programme CHARMm¹⁶, which showed the lowest energy conformation when the γ -hydroxyl of Thr 56 was 3.5 Å from the γ -hydroxyl of Ser 36 (Fig. 4d). If an internal hydrogen bond formed in this location, it would be expected to increase the (thermo-)stability^{17,18} and resistance to degradation of the variant protein, which could contribute to amyloidogenesis.

Definition of the actual effect of the Thr-56 variant on the

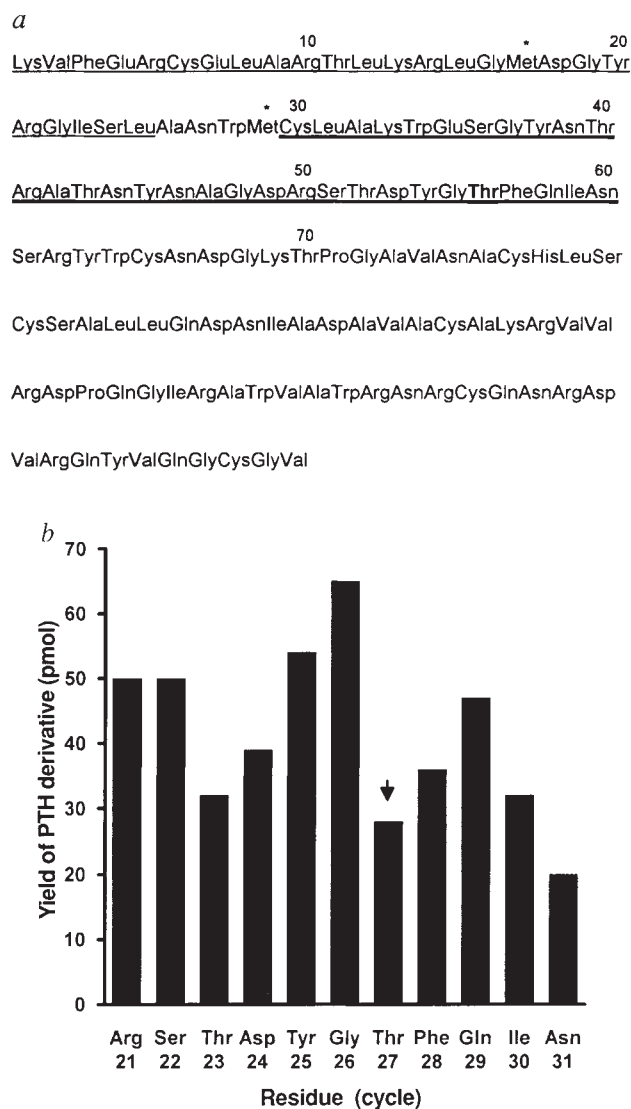
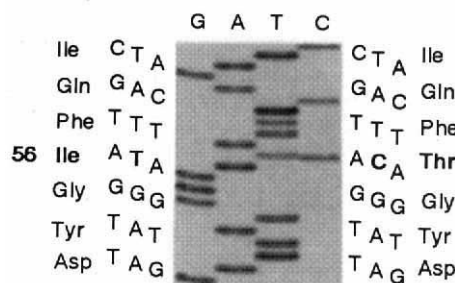


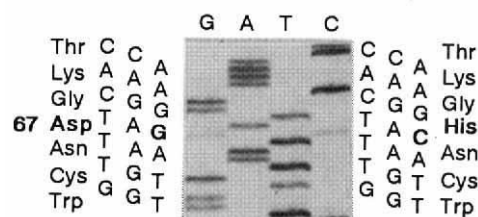
FIG. 3 **a** and **b**, Amino-acid sequence of the protein subunit of amyloid fibrils from the proband in family 1. Aliquots of the isolated fibril preparation were separated by SDS-PAGE and transferred to Problott membrane (Applied Biosystems). The major band at M_r 15K was sequenced using fast-cycle chemistry in a modified Applied Biosystems 477A (ref. 25). **a**, Complete amino-acid sequence of mature lysozyme with substitution of Thr for Ile at position 56. The amino-terminal sequence, which is single-underlined, was obtained from the fibril subunit up to 25 cycles. The yield was confirmed as representative of the total protein by automated amino-acid analysis of an equivalent sample on an ABI 420A (Applied Biosystems). The fibrils completely resisted proteolysis by trypsin under native conditions, and by lysyl endopeptidase in the presence of 2 M guanidine, 2 M urea or 0.1% w/v SDS. They were therefore treated with cyanogen bromide in 70% formic acid²⁶. The resuspended peptide mixture was subjected directly to automated sequencing as described. The sequence is single-underlined, and is exactly the reported sequence of human lysozyme except for the presence of Thr (bold type) at cycle 27 of the largest fragment, corresponding to residue 56 of the mature protein. The Met residues at which cleavage by cyanogen bromide takes place are indicated by an asterisk. **b**, Yields of phenylthiohydantoin (PTH)-amino acids at cycles 21–31 of the largest

c

Family 1



Family 2



fragment, corresponding to residues 50–60 of the mature protein; the variant residue Thr is indicated by an arrow. Ile, the wild-type residue, was detected at position 56 only at the background level (~3 pmol). **c**, Nucleotide sequence of part of exon 2 of the lysozyme gene from the probands with amyloidosis. Genomic DNA from the proband in family 1 was isolated from liver²⁷ which had been stored frozen at -80°C since autopsy in 1984, and from whole blood from the proband in family 2 (ref. 28). Exon 2 of the lysozyme gene²⁹ was amplified by polymerase chain reaction (PCR) as described³⁰, using the following primers located in the flanking introns: 5'-AGTCACTTAGTGTGCTGTTT and 5'-ACCAGATTGGTCAAATATTAG. The product was purified and sequenced directly³¹, primed with the 5'-flanking amplification primer. The proband in family 1 was heterozygous for a single base substitution that alters the codon at position 56 in the mature protein from Ile to Thr. The proband in family 2 was heterozygous for a single base substitution that alters the codon at position 67 in the mature protein from Asp to His. The remainder of the coding region in both individuals was identical to the published sequence of the human lysozyme gene²⁹. Exon 2 amplified by PCR from the DNA of 100 unrelated adults undergoing prospective health screening was negative in allele-specific hybridization with probes for both mutations, indicating that these are not common polymorphisms.

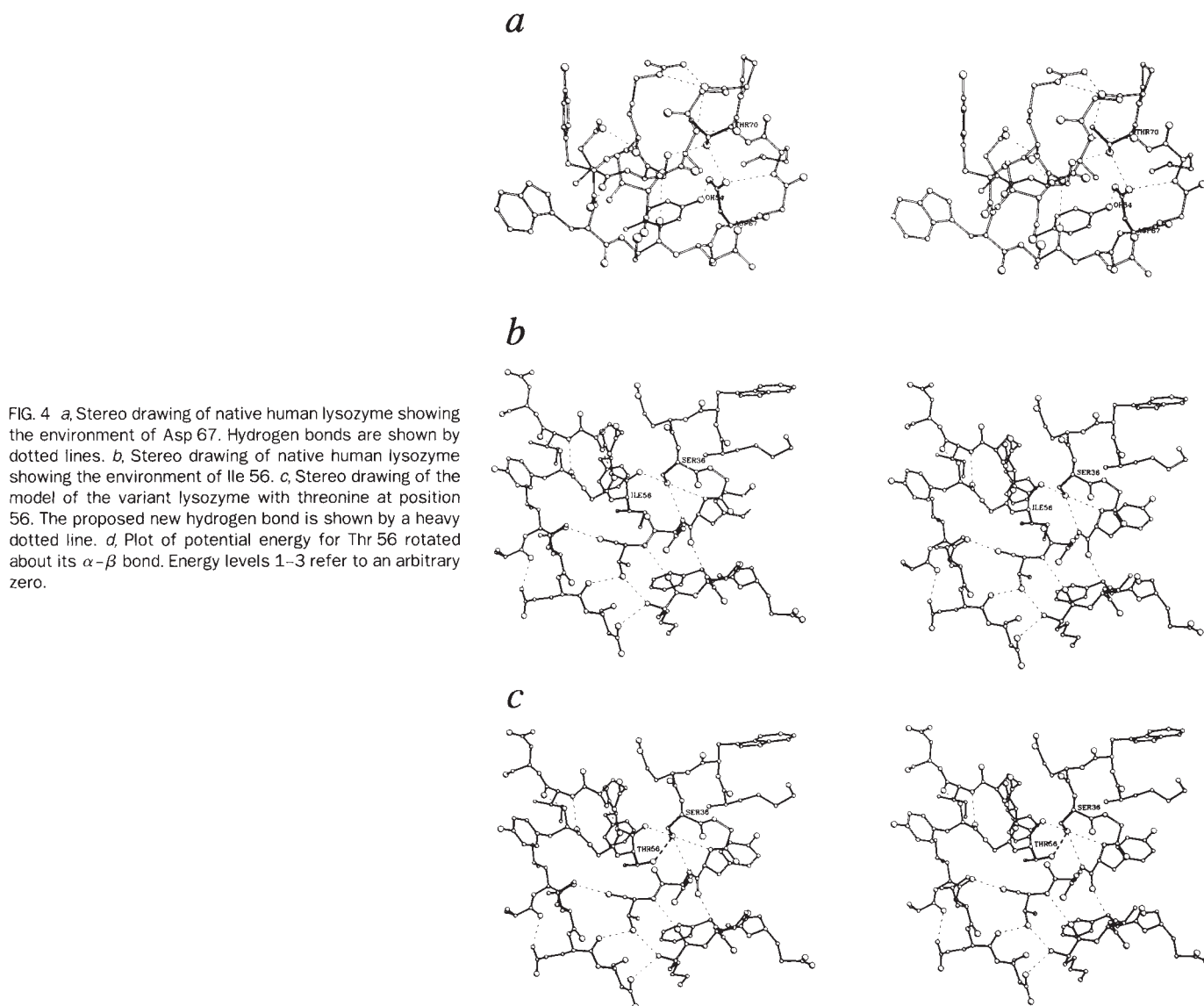
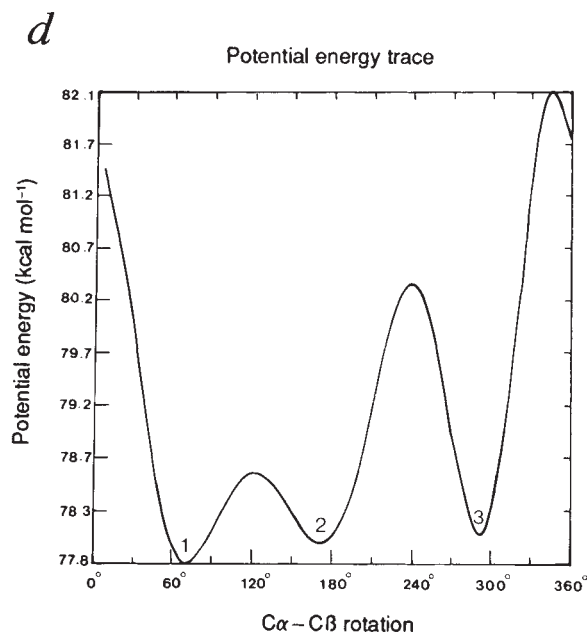


FIG. 4 *a*, Stereo drawing of native human lysozyme showing the environment of Asp 67. Hydrogen bonds are shown by dotted lines. *b*, Stereo drawing of native human lysozyme showing the environment of Ile 56. *c*, Stereo drawing of the model of the variant lysozyme with threonine at position 56. The proposed new hydrogen bond is shown by a heavy dotted line. *d*, Plot of potential energy for Thr 56 rotated about its α - β bond. Energy levels 1-3 refer to an arbitrary zero.



molecular structure of lysozyme, and confirmation of the prediction of the His-67 change, require crystallization and analysis of both variant lysozymes. Although the crystal structures of two other amyloidogenic proteins, transthyretin and β_2 -microglobulin, are known, both normal proteins can form fibrils¹⁹. In contrast, wild-type lysozyme is not amyloidogenic and structural analysis of the present variants and of lysozyme fibrils may therefore be informative about general mechanisms of amyloid fibrillogenesis. □

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Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch

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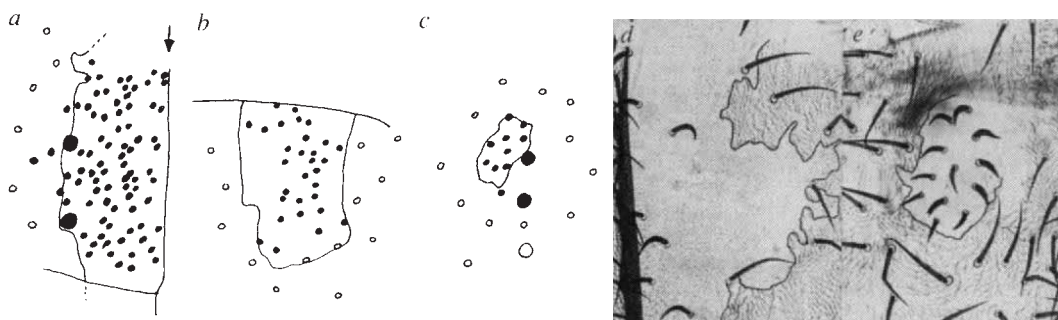
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DURING neurogenesis in *Drosophila*, groups of equipotential, neurally competent cells choose between epidermal and neural fates^{1–4}. Notch, a phylogenetically conserved transmembrane protein^{5–9}, may act as a receptor^{4,10} in a lateral signalling pathway in which a single neural precursor is chosen from each group and the neural fate of the other cells is inhibited, causing them to differentiate into epidermis^{11–13}. Possible intracellular transduction events mediating signals from Notch are, however, unknown. *shaggy* is also required for the lateral signal^{4,14} and encodes serine/threonine protein kinases^{15,16} with homology to the glycogen synthase kinase-3 (GSK-3) enzymes¹⁷ that act in signal transduction pathways in vertebrates¹⁷. We report here that, in

transgenic flies, GSK-3 β can substitute for *shaggy*, and we also present a study of epistatic relationships between *shaggy* and gain and loss of function alleles of Notch. The results indicate that *shaggy*/GSK-3 is part of a signalling pathway downstream of Notch.

On the thorax, each equivalence group is composed of about six cells and in *Delta* (*Dl*) or *Notch* (*N*) mutants every cell differentiates a sensory bristle¹⁴, whereas in *shaggy* (*sgg*) mutants an average of three cells do so⁴. All cells initially have signalling and receiving properties and express the receptor, *N*, as well as *Dl*, another transmembrane protein thought to be the signal and a ligand for *N* in this process^{4,10}. Cells with little or no *N* protein have an increased ability to signal (through *Dl*) and they always inhibit adjacent wild-type cells^{4,18} (Fig. 1a). A feedback loop may thus exist within each cell that couples reception and signalling. Singling out one cell as the neural precursor may rely on randomly fluctuating amounts of *N* such that a cell expressing less *N* signals more efficiently, gains a greater advantage and inhibits adjacent cells⁴. Both signalling and receiving abilities are simultaneously impaired in cells mutant for *sgg* that behave like double mutant *N Dl* cells^{4,18}. *shaggy* is not, however, a physical component of the signal: like mutant *N* cells, cells doubly mutant for *N* and *sgg* send a strong inhibitory signal (Fig. 1a–c). Thus mutant *N* cells do not require *sgg* to inhibit their neighbours and *sgg* is therefore dispensable for the signal. Because cells doubly mutant for *N* and *sgg* strongly inhibit their neighbours but *N*⁺ *sgg*[–] cells produce only a poor signal⁴, it follows that absence of *sgg* decreases the cell's

FIG. 1 Phenotype of *sgg* and *N* mutant and double mutant thoracic clones and the capacity of mutant cells to inhibit adjacent wild-type cells. Mutant bristles are curved and mutant epidermal hairs are tufted. On the drawings, the closed circles indicate mutant bristles and open circles wild-type bristles. a, Clone mutant for *N*^{ts1} showing bristle hyperplasia (arrow indicates the midline). In mosaics, where mutant and wild-type cells are juxtaposed, mutant *N* cells send a strong inhibitory signal such that their wild-type neighbours are forced into the epidermal fate⁴. Thus, along the borders of *N*^{ts1} cells, only 2% of bristles are wild type (number of bristles scored 121). (If the choice is random, marked bristles are on the border 50% of the time⁴.) b, Clone mutant for *sgg*^{D127}. In this case 31% of the bristles along the mosaic borders are wild-type in genotype (*n* = 154). c, Clone doubly mutant for *sgg*^{D127} and *N*^{ts1}. Cells of this genotype behave like those mutant for *N*^{ts1} alone and neighbouring wild-type cells adopt the epidermal fate: only 1% wild-type bristles are found on the mosaic border (*n* = 120). d, Photograph of a clone (outlined) homozygous mutant for *Ax*^{SX1}, a gain of function allele of *N* in which most of the cells take up the epidermal fate¹⁸. The density of bristles can be measured on the differentiated cuticle by counting the number of intervening epidermal cells. An average of 8.9 ± 0.38 cells separate bristles of this genotype (74 bristle



pairs were scored). In wild-type flies, bristles are separated by 4.9 ± 0.12 epidermal cells¹⁴. In contrast, in clones mutant for *sgg*^{D127} most cells take up the neural fate (see Figs 1b and 3e) and the average distance between bristles is 2.7 ± 0.23 (*n* = 118). e, Photograph of a clone (outlined) doubly mutant for *sgg*^{D127} and *Ax*^{SX1}; the density of bristles is 2.5 ± 0.25 (*n* = 101). These clones thus display a *sgg* phenotype and *sgg* is therefore epistatic over *Ax*.

METHODS. Mutant clones were induced and flies prepared according to the method described in ref. 4, in flies of the following genotypes: (1) *Ax*^{SX1} *f*^{36a} / *Dp*(3;Y;1)M2, *mwh*⁺ *y* *v*; *mwh*; (2) *sgg*^{D127} *Ax*^{SX1} *f*^{36a} / *Dp*(3;Y;1)M2, *mwh*⁺ *y* *v*; *mwh*; (3) *N*^{ts1} *f*^{36a} / *Dp*(3;Y;1)M2, *mwh*⁺ *Dp*(1;1)Co, *N*⁺ *v*; *mwh*; (4) *sgg*^{D127} *N*^{ts1} *f*^{36a} / *Dp*(3;Y;1)M2, *mwh*⁺ *Dp*(1;1)Co, *N*⁺ *v*; *mwh*; and (5) *sgg*^{D127} *f*^{36a} / *Dp*(3;Y;1)M2, *mwh*⁺ *y* *v*; *mwh*. Animals of genotypes 3, 4 and 5 were grown at 29 °C.

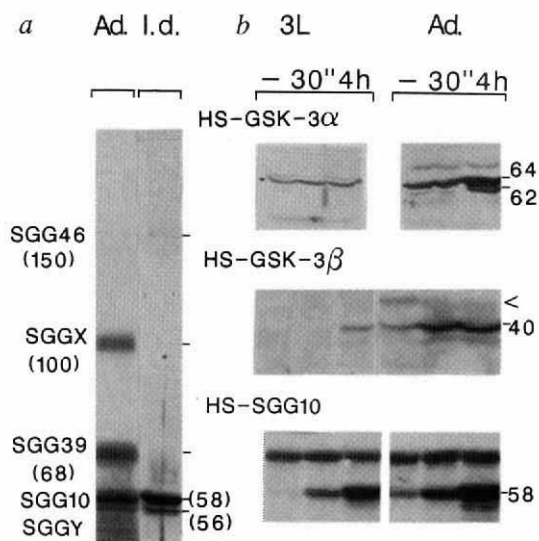
FIG. 2 Expression of the *sgg* kinases and the rat gene homologues, the GSK-3 enzymes²¹, in wild-type and transgenic lines. **a**, Western blot analysis of proteins from adult flies (first lane) reveals five forms of *sgg* proteins derived from alternative splicing, each containing the catalytic domain but with different N or C terminals²⁰. Only three are present in imaginal wing discs of third instar larvae (second lane). Sequence comparison and alignment of the *sgg* kinases and the rat GSK-3 kinases (not shown) suggests that SGG10 is the most closely related to the mammalian enzymes. GSK-3 α and GSK-3 β are similar enzymes encoded by different genes²². GSK-3 β is marginally closer to SGG10 than is GSK-3 α when considering a 312 amino-acid region including the kinase catalytic domain (85% amino-acid identity compared to 83%; whereas GSK-3 α and GSK-3 β are 91% identical over the same region). But SGG10 (as well as the other *sgg* kinases) possesses an additional domain rich in Gly, Ala and Ser at the C terminus (GAS domain)¹⁶, not present in the GSK-3 enzymes. GSK-3 α also differs from GSK-3 β by a Gly-Ser rich segment in an N-terminal region. **b**, Transgenic lines containing HS-SGG10, HS-GSK-3 α or HS-GSK-3 β constructs, were analysed for expression of the respective proteins. In each case (larvae, first three lanes, adults, next three lanes), the respective specific immunoreactive proteins were induced (or showed an increased accumulation) 30 min and 4 h after heat-shock treatment. Some other faint immunoreactive bands were also found using each of the anti-GSK-3 immunosera. They were either crossreactions to *Drosophila* proteins or were specific for the GSK-3 transgenes. The accumulation of a heat-inducible SGG10 protein of the expected size (58K) was quite strong relative to that of the SGG39 protein synthesized from a minigene that had been simultaneously introduced. Heat-shock induction of lines transformed with HS-GSK-3 β resulted in the synthesis of a 40K GSK-3 β protein migrating below purified GSK-3 β (obtained from J. Woodgett; not shown). A small amount (arrowhead) of a protein of the expected M_r of 47K was nevertheless detected. Flies transformed with HS-GSK-3 α constructs showed induction of a 62K GSK-3 α protein and the constant presence of a 64K band. Both species were larger than the predicted M_r of 51K for GSK-3 α . Expression of the same GSK-3 α cDNA in COS cells also resulted in the production of a GSK-3 α immunoreactive band of 59K in addition to the predicted 51K protein²². The nature of such possible modifications of GSK-3 proteins when expressed in flies was not investigated.

METHODS. HS-SGG10, HS-SGG46, HS-GSK-3 α and HS-GSK-3 β chimaeric gene constructs were made by inserting corresponding complementary DNA sequences into the P-element transformation and expression vector pCaS-

signalling ability only in the presence of *N* molecules. This argues that *sgg* may only modulate the cell's signal through an interaction with the *N* molecule that in turn affects the activity of *Dl*.

In contrast, *sgg* is an indispensable component of signal reception. The dominant *Abruptex* (*Ax*) alleles of *N* (ref. 19) cause a phenotype opposite to that of loss of function alleles: most or all cells adopt the epidermal fate (Fig. 1d). *Ax* molecules, however, do not function as constitutive receptor proteins: their phenotype is suppressed by lowering the dosage of *Dl*, the gene postulated to encode the ligand, and furthermore *Ax* cells are unable to differentiate as epidermis in the absence of *Dl*, showing that they require the ligand to transduce the signal¹⁸. The *Ax* proteins seem to be hyperactive molecules that display a greater affinity for the ligand. Thus *Dl* is epistatic over *Ax*: the double mutant *Ax Dl* clones differentiate as neural cells. Thus genes acting upstream of the signal, in addition to genes acting downstream of the receptor, would be epistatic over *Ax*. Indeed, *sgg* is epistatic over *Ax*: cells doubly mutant for *Ax* and *sgg* show a *sgg* phenotype of neural hyperplasia (Fig. 1e). Because *sgg* is dispensable for the signal, this result indicates that *sgg* is required after *N* in the inhibitory pathway and may be a component of signal transduction.

At least five protein kinases are produced at the *sgg* locus²⁰ (SGG10, SGG39, SGG46, SGGX and SGGY, Fig. 2a) and in wild-type imaginal discs three proteins are detected including SGG10 (Fig. 2a). Previous experiments using *sgg* minigenes showed that SGG10 probably effects most of the different *sgg* functions²⁰. To test whether SGG10 and GSK-3 can rescue the bristle hyperplasia, heat shock-inducible constructs expressing SGG10 (HS-SGG10) and the two forms of rat GSK-3 (ref. 22),



per HS. The 2.0 kb *Bgl*III-*Xba*I fragment of cDNA NB10, the 3.9 kb *Bgl*III-*Xba*I fragment of cDNA NB46 and the complete cDNAs of 2.15 kb and 2.25 kb for GSK-3 α and GSK-3 β , were cloned^{20,22}. Transformed lines (P[w⁺, (HS)]) were obtained as described previously²⁰. Selected males of the genotypes *w¹¹¹⁸/Y*; HS-GSK-3 α , *w¹¹¹⁸/Y*; HS-GSK-3 β , and *sgg^{D127}/Y*; *mini39*/HS-SGG10 (*mini39* is a minigene expressing SGG39 used to rescue mutant lethality) were exposed for 30 min to 40 °C in tubes containing a drop of water (larvae) or in fly culture vials (adults) and allowed to recover at room temperature for the time indicated. Untreated animals served as controls. Total protein extracts of samples from five animals, were analysed by Western blotting using 7% SDS-PAGE and rabbit immunoserum raised against GSK-3 α or GSK-3 β ²² and the monoclonal antibody Mab2G2C5, raised against *sgg* proteins as described previously²⁰. Artefactual staining at the bottom of the gel (first lane in A) results from accumulation of stacked proteins in 7% PAGE. Two independent insertion lines were analysed in each case.

GSK-3 α (HS-GSK-3 α) and GSK-3 β (HS-GSK-3 β), were introduced by P-element-mediated transformation and shown to be expressed in transgenic flies (Fig. 2b). When assayed for their ability to rescue the zygotic lethality of *sgg^{D127}*, a protein null allele, and *sgg^{b12}*, a lethal allele lacking expression of SGG10, SGGY and SGG39 (ref. 20), HS-SGG10 resulted in viable adults displaying wild-type morphology after maximal heat-shock treatment (Fig. 3a, b). A lower heat-shock regimen allowed the development of mutant imagos showing the bristle hyperplasia expected of a hypomorphic condition (Fig. 3c). This phenotype is very similar to that of animals mutant for *N^{ts1}*, a conditional allele of *N*, when exposed to restrictive temperature during early pupal periods²¹. Under conditions of maximal heat treatment, HS-GSK-3 β but not HS-GSK-3 α , was able to effect some rescue of animals mutant for *sgg^{b12}* but not *sgg^{D127}* (Fig. 3a, d). Rescue of *sgg^{D127}* mutant clones in the imaginal wing disc was also assayed to circumvent the requirements for *sgg* in early development. Both HS-SGG10 and HS-GSK-3 β completely rescued the bristle hyperplasia (Fig. 3f, g). Therefore GSK-3 β can replace *sgg* during the epidermal-neural cell fate decision, but is less effective at other stages of the life cycle at which *sgg* is required²³.

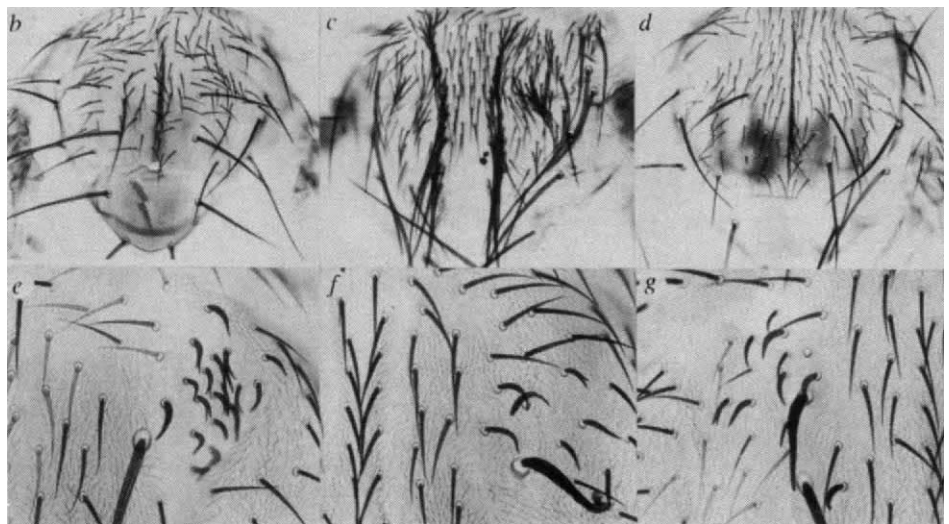
We conclude that, under our experimental conditions, GSK-3 β can phosphorylate *sgg* substrates in the wing disc but that there may be a biochemical difference between the two forms of GSK-3 (Figs. 2 and 3). Our observations place *sgg*/GSK-3 downstream of *N* in a signal transduction pathway (Fig. 4). The endpoint of this signal would lead to a repression of *achaete* and *scute*, the genes conferring neural potential²⁴. It has been shown that *achaete* and *scute* are first expressed in all cells of the equivalence group but are later repressed in the inhibited cells^{2,25}. In vertebrates, GSK-3 may modulate the activity of

FIG. 3 Rescue of *sgg* mutants in transgenic lines containing HS-SGG10, HS-SGG46, HS-GSK-3 α and HS-GSK-3 β chimaeric gene constructs. *a*, Rescue of the larval lethality of *sgg*^{b12} mutants containing single copies of the constructs. *sgg*^{b12} is associated with a chromosomal inversion that prevents expression of SGG10, SGGY and SGG39 but allows that of SGG46 and SGGX. *b*, Maximal treatment with HS-SGG10 effected complete rescue to a wild-type phenotype. Control experiments without heat shock gave no rescue: a few poorly developed pupae resulted from low basal expression. A single heat shock per day rescued about 50% of the animals with no pattern defects. *c*, After a single heat shock every other day, most animals developed to pharate adults displaying bristle hyperplasia, ectopic wing bristles, a notal cleft and small rough eyes. Stronger rescue was associated with two HS-SGG10 lines. Under conditions of maximal heat shock, HS-SGG10 was also able completely to rescue *sgg*^{D127}, showing that when present in sufficient amounts SGG10 alone allows the segregation of normal numbers of bristles. The possible contribution of SGG46, also expressed in imaginal discs (Fig. 2a) was assayed with HS-SGG46, but no signs of rescue of either *sgg*^{b12} or *sgg*^{D127} were observed. Limited rescue of *sgg*^{b12} was achieved with HS-GSK-3 β , when compared to HS-SGG10: pharate adults or adult escapers showed a normal bristle pattern (*d*) or an increased bristle density. No rescue was achieved with HS-GSK-3 α . It is possible that regulation, subcellular localization, or the kinetic parameters of the phosphorylation reaction of target substrates, may distinguish the two GSK-3 enzymes because the major structural differences between them reside outside the kinase catalytic domain²². Alternatively the GSK-3 α enzyme is adversely modified in the fly (Fig. 2). Rescue of thoracic *sgg*^{D127} clones could not be achieved with HS-GSK-3 α (*e*) or HS-SGG46, but expression of HS-SGG10 (*f*) and HS-GSK-3 β (*g*) resulted in normal bristle spacing. Marked bristles are curved and were separated by 4.0 ± 0.24 epidermal cells, $n=47$, see Fig. 1. METHODS. Heat shocks were applied by putting vials of fly cultures into a

a

The extent of mutant rescue by HS-SGG/GSK-3 transgenes: figures represent the number of independent transgenic lines tested

Genotypes	larval lethality (not rescued)	Pupariation and some pupal development	Pharate adults including viable escapers	Viable adults displaying a normal pattern
<i>sgg</i> ^{b12} /Y; HS-SGG10/+				4
<i>sgg</i> ^{b12} /Y; HS-SGG46/+	7			
<i>sgg</i> ^{b12} /Y; HS-GSK-3 β /+	1	7	3	
<i>sgg</i> ^{b12} /Y; HS-GSK-3 α /+	6			



water bath. Cultures contained the progeny of crosses between males carrying heat-shock constructs, with either *sgg*^{b12}/FM7 or *sgg*^{D127} w s/FM7 females. Rescue was quantified with respect to siblings²⁰, and compared to control crosses without heat shock. We found that periodic heat shocks were necessary to obtain viable adults, presumably because of a continuous requirement for *sgg* throughout larval stages. Maximal heat-shock treatment consisted of 15 min at 40 °C twice a day for a period of 6 days. This induced considerable lethality in all classes of progeny. Mutant clones were induced between 48 and 72 h after egg laying as in ref. 4, in *sgg*^{D127} w f^{36a}/y w^{67c23}; P[w⁺, (HS)]/P[w⁺, (HS)] flies. Five heat shocks (30 min at 40 °C) were given every 8 or 16 h after irradiation.

transcription factors by phosphorylation^{26,27} and so perhaps targets for *sgg* may be found among transcriptional regulators of the neural fate¹.

These results reinforce the case for a role of *N* as a cellular receptor. *Notch* homologues are present in many species and are expressed in many tissues⁷⁻⁹. The *N*-mediated lateral signalling

mechanism may be a means of creating the patterns of spaced elements frequently seen in nature, such as that of bristles in insects and on a finer scale of allowing two equivalent cells to take up alternative fates, in a manner like that of two similar genes in the nematode, *lin-12* and *glp-1*²⁸. The mechanism by which intracellular signalling is initiated after ligand binding of *N* awaits investigation. □

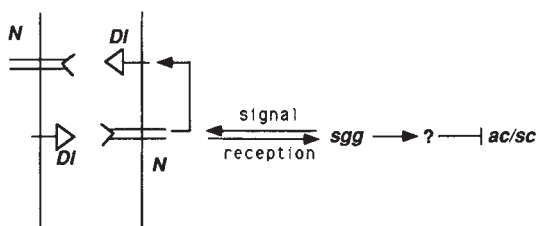


FIG. 4 Scheme depicting the roles proposed for the genes *N*, *DI* and *sgg* during lateral inhibition. *Delta* is visualized as a signal protein that acts as a ligand¹⁰ for a receptor, *N*⁴. *shaggy* acts downstream of *N* in the transmission of the signal resulting in the repression of *achaete* and *scute* but is upstream of *N* in the regulation of a cell's own ability to signal because the cell's signalling and receiving abilities are coupled.

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Interactions of three domains distinguishing the Ras-related GTP-binding proteins Ypt1 and Sec4

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THE genes *SEC4* and *YPT1* encode Ras-related GTP-binding proteins in the yeast *Saccharomyces cerevisiae*. Ypt1 is necessary for vesicular transport from the endoplasmic reticulum to the Golgi¹⁻⁴, whereas Sec4 is required for fusion of post-Golgi secretory vesicles to the plasma membrane⁵. Recently, three structural domains have been proposed to specify the stage in cellular transport at which members of the Sec4/Ypt1/Rab family act: the effector domain⁶, the C-terminal hypervariable region⁷, and a region corresponding to loop 7 in the structure of p21^{ras} (ref. 8). Here we use Sec4/Ypt1 chimaeras to show that these three regions cooperate to specify Ypt1 function and that the C-terminal hypervariable region is needed for Ypt1 localization to the Golgi. Unexpectedly, we found that a single chimaera can function as either Ypt1 or Sec4 without missorting carboxypeptidase Y or invertase.

The effector domain of the *ras* protein (Ras), first identified in a screen of point mutations that abolish the transforming potential of otherwise activated *ras* alleles⁹, interacts with a GTPase-activating protein (GAP), which possibly relates to the effector function of this region¹⁰⁻¹². Point mutations in the effector region of Ypt1 are deleterious, both to its function in yeast protein transport, and to its stimulation by a mammalian Ypt1-GAP activity⁶. To examine the role of the effector domain in defining the specificity of Sec4 and Ypt1 function, we constructed chimaeras containing reciprocal exchanges of this region, designated *SEC4-EF*^{YPT} and *YPT1-EF*^{SEC4} (Figs 1 and 2). Like the *YPT1* control, *YPT1-EF*^{SEC4} fully complemented a *YPT1* deletion, but not a *SEC4* deletion (Fig. 2). *SEC4-EF*^{YPT} however, complemented neither the *SEC4* deletion, nor the *YPT1* deletion (Fig. 2). By this assay, therefore, the effector domain of *SEC4* is uniquely required for its function, whereas *YPT1* can function with the *SEC4* effector domain in place of its own.

Sec4 is associated with post-Golgi secretory vesicles and the plasma membrane¹³, whereas Ypt1 is found on endoplasmic reticulum(ER)-to-Golgi vesicles and on the yeast Golgi apparatus^{1,14}. The C-terminal hypervariable region functions as a signal for the localization of mammalian Rab5 and Rab7 to early and late endosomes, respectively⁷. We examined the effect of exchanging the analogous hypervariable regions of Sec4 (HV^{SEC4}) and Ypt1 (HV^{YPT}) on their function and localization.

Surprisingly, both proteins function well with the alternative hypervariable region: the *SEC4-HV*^{YPT} construct fully complemented a deletion of *SEC4*, but not *YPT1*, and the *YPT1-HV*^{SEC4} chimaera fully complemented a deletion of *YPT1*, but not *SEC4* (Fig. 2).

Using immunofluorescence microscopy we examined the role of the HV^{YPT} sequence in the subcellular localization of Ypt1. The enlarged Golgi-like structures that develop upon incubation of a *sec7-1* mutant at 37 °C label intensely with Ypt1 antibody^{1,15}, which is evident as a distinct punctate immunofluorescent pattern. The control *sec7-1* strain containing wild-type *SEC4* and *YPT1* gave punctate Ypt1 staining as expected, whereas Sec4 staining was more diffuse (Fig. 3a). This demonstrates that wild-type Sec4 does not appreciably associate with the enlarged *sec7-1* Golgi structures and is consistent with the earlier localization of Sec4 to post-Golgi compartments¹³. When the *Sec4-HV*^{YPT} chimaera was present as the only source of *SEC4* in a *sec7-1* strain, however, the Sec4 antibody gave bright punctate staining which was largely coincident with the punctate Ypt1 staining (Fig. 3b). This suggests that the HV^{YPT} domain in this chimaera allows it to associate efficiently with the Golgi. Furthermore, a chimaera in which the hypervariable domain of *YPT1* is replaced with that of *SEC4* (*YPT1-HV*^{SEC4}), did not show the punctate staining typical of Ypt1 in a *sec7-1* strain, but rather the diffuse staining typical of Sec4 in a *sec7-1* strain (results not shown). The HV^{YPT} domain therefore constitutes a Ypt1 localization domain. Although the high-efficiency localization of Ypt1 aids its function (see later), it is not essential for Ypt1 function. Presumably even at normal expression levels, sufficient Ypt1-HV^{SEC4} can associate with the proper membrane to promote normal Ypt1 function.

A region of Ypt1 corresponding to loop 7 in the crystal structure of Ras (L7^{YPT}; Fig. 1) has been shown to confer Ypt1 function on a *SEC4* gene⁸. Consistent with these results, we found that *SEC4-L7*^{YPT} can complement a *YPT1* deletion, although with poor efficiency (see Fig. 2 legend). The reciprocal exchange allele (*YPT1-L7*^{SEC4}) efficiently complements the deletion of *YPT1*, but not *SEC4*. Despite the fact that the L7^{YPT} sequence alone can confer Ypt1 function onto *SEC4*, the L7^{YPT} sequence is not uniquely required for Ypt1 function.

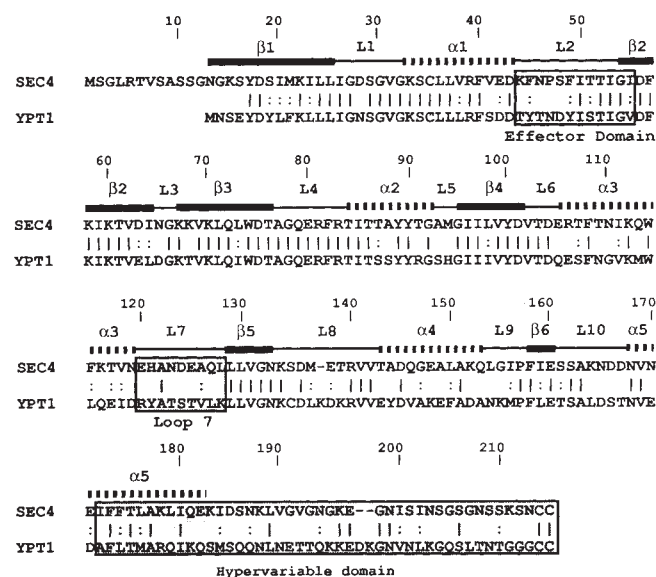


FIG. 1 Sequence and structure alignment of Sec4 and Ypt1. Secondary structure elements, determined from the Ras crystal structure¹⁷, are placed according to the alignment of Sec4 with Ras shown previously⁹. Numbering of Sec4 residues is shown above the structure alignment. Sequence identities are marked with solid lines and conservative changes are marked with a colon. The effector domain, loop 7 and the hypervariable region are shaded.

The *SEC4-L7^{YP}* construct complemented a *YPT1* disruption inefficiently, but addition of the *HV^{YP}* domain to yield the double-exchange allele *SEC4-(L7, HV)^{YP}* allowed efficient complementation at 25 °C (Fig. 2), presumably by facilitating localization to the site of Ypt1 action. Growth failed however, at 37 °C or 14 °C. The reciprocal double-exchange allele (*YPT1-(L7, HV)^{S4}*) was incapable of rescuing the *YPT1* deletion (Fig. 2). *L7^{YP}* and *HV^{YP}* therefore act in a cooperative fashion to facilitate the function of Ypt1, and their replacement with Sec4 sequences leads to a synergistic loss of function. This functional interaction may reflect physical proximity, because the analogous domains of Ras are thought to be in adjacent regions of the membrane-proximal surface of the protein^{16,17}.

Addition of the Ypt1 effector domain (EF^{YP}) to the *SEC4-(L7, HV)^{YP}* construct (Fig. 2) to create the triple-exchange allele *SEC4-(EF, L7, HV)^{YP}* allowed complementation of the *YPT1* deletion at 25 °C, but also allowed these cells to grow normally at 37 °C (but poorly at 14 °C). The EF^{YP} domain thus suppresses the temperature-sensitivity of the *SEC4-(L7, HV)^{YP}* chimera in a *YPT1* deletion strain. Replacing the effector domain of *YPT1* with that of *SEC4* resulted in a complete loss of *YPT1* complementing activity when combined with replacement of either loop 7 (*YPT1-(EF, L7)^{S4}*) or the hypervariable domain (*YPT1-(EF, HV)^{S4}*). In summary, none of these three regions alone is essential to the functional specificity of Ypt1, but

replacement of any two of these three regions is synergistically deleterious to Ypt1 function.

Several bypass suppressors of *YPT1* have been reported¹⁸⁻²¹. These suppressors encode proteins unrelated to Ypt1 and allow slow growth of Ypt1-depleted cells and only partially restore the transport and processing of invertase and carboxypeptidase Y. In contrast, the Sec4/Ypt1 chimaeras described here efficiently restore growth and protein transport to cells depleted of Ypt1 (Figs 2 and 4). Furthermore, the same sequences that cooperate to promote Ypt1-complementing activity when introduced into Sec4 also negatively cooperate in loss of activity when they are replaced with Sec4 sequences in Ypt1. Therefore it is likely that we are observing gain and loss of normal Ypt1 function, rather than a bypass of its requirement.

An unexpected finding was that the *SEC4-(L7, HV)^{YP}* construct not only substituted for *YPT1* at 25 °C, but also fully substituted for *SEC4*. Extending this observation, we found that the *SEC4-(L7, HV)^{YP}* chimera could complement the simultaneous deletion of both *SEC4* and *YPT1* (not shown). These *sec4Δ, ypt1Δ, SEC4-(L7, HV)^{YP}* cells were slower growing than either single disruption bearing the chimera, and were temperature-sensitive (*ts*⁻) and cold-sensitive (*cs*⁻), as with the *ypt1Δ* strain. The slower growth may reflect an additive effect of partial defects at two stages of the transport pathway or that the level of chimaeric protein, expressed from a single gene, is

FIG. 2 Genetic analysis of the ability of *SEC4* and *YPT1* chimaeras to complement deletions of these genes: (+) indicates normal growth, (-) indicates no detectable growth, (-/+) indicates very slow growth; (% Inv. secr.) indicates the percentage of external invertase after 90 min in low-glucose medium at 25 °C. Abbreviations are: EF, effector domain; L7, loop 7 and HV, hypervariable domain.

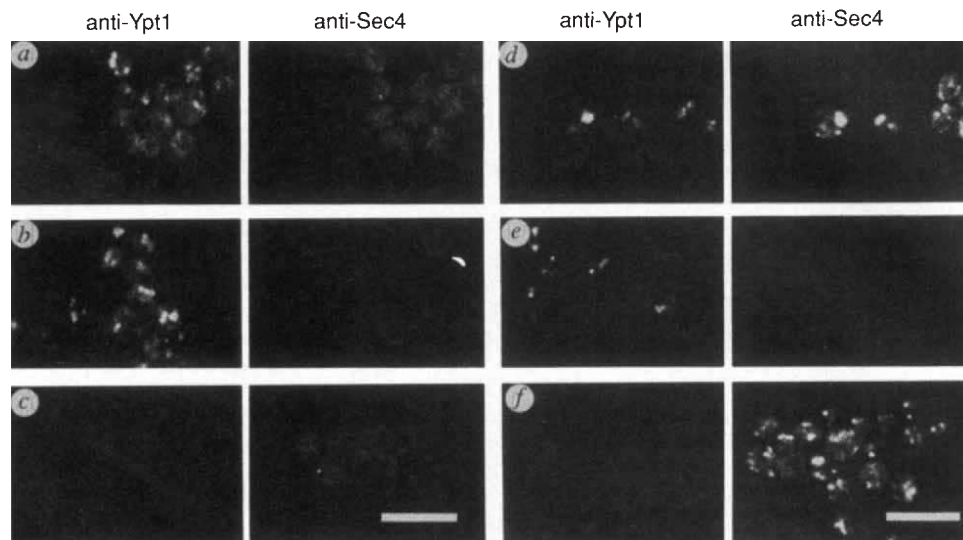
METHODS. Shuttle-vectors were prepared in the pUC118 phagemid containing *LEU2* and either *SEC4* (pNB359) or *YPT1* (pNB420). The effector domain and loop 7 chimaeras were prepared by oligo-directed mutagenesis according to ref. 22. Residues that were exchanged in each region are indicated in Fig. 1. Exchange of the hypervariable domains was prepared by first inserting a *SacI* site at position 172 in *SEC4* and position 161 in *YPT1*. The incorporation of this restriction site results in an Ile-to-Leu change at residue 172 in Sec4 and an Ala-to-Leu change at residue 161 in Ypt1. Both *sec4-L172* and *ypt1-L161* were tested for function and both showed wild-type complementation. Using these new sites, fragments containing the hypervariable domains were exchanged between the two genes. All of the chimaeric genes were introduced into each of two diploid yeast strains containing heterozygous deletions of either *SEC4* or *YPT1*. NY810 (*a/α;ura3-52/ura3-52; leu2-3,112/leu-3, 112; sec4Δ::URA3/SEC4*) has one copy of *SEC4* in which the region from amino acids 62 to 189 (*EcoRV* to *HindIII*) was replaced with the *URA3* gene. NY921 (*a/α; ura3-52/ura3-52; leu2-3,112/leu2-3,112; his3-Δ200/his3-Δ200; ypt1Δ::HIS3/YPT1*) contains a copy of *YPT1* in which the region from amino acids 16 to 191 (*EcoRI* to *HincII*) was replaced with the *HIS3* gene. The plasmids were cleaved within the *LEU2* gene to target chromosomal integration at the *LEU2* locus. Diploids were transformed by the lithium acetate method²³. Transformants were colony-purified, sporulated and tetrads dissected with a micromanipulator on YPD plates. The plates were grown at 25 °C and the haploid progeny analysed by replica plating for the presence of the integrated chimera (scored as *leu*⁺) for the presence of a deleted *SEC4* or *YPT1* (scored as *ura*⁺ for *sec4Δ* and his⁺ for *ypt1Δ*) and for growth on YPD at 14 °C and 37 °C. Tetrad analysis of non-functional chimaeras showed 2:2 segregation of viability and all of the viable spores contained the wild-type copy of *SEC4* or *YPT1*. All of the chimaeras that failed to complement the *SEC4* deletion were also tested for suppression of the temperature-sensitive *sec4-8* mutation. Likewise, those that failed to complement the *YPT1* deletion were tested for sup-

	Effector domain	Loop 7	Hypervariable domain	Complementation of:				Complementation of:			
				Sec4 ^Δ at:	at:	% Inv. secr.	Ypt1 ^Δ at:	at:	% Inv. secr.	at:	% Inv. secr.
				25 °C	14 °C	37 °C		25 °C	14 °C	37 °C	
SEC4				+	+	+	98	-	-	-	-
Sec4-HV ^{YP}				+	+	+	96	-	-	-	-
Sec4-L7 ^{YP}				+	+	+	96	-	+	-	-
Sec4-(L7, HV) ^{YP}				+	+	+	93	+	-	-	94
Sec4-EF ^{YP}				-	-	-	-	-	-	-	-
Sec4-(EF, HV) ^{YP}				-	-	-	-	-	-	-	-
Sec4-(EF, L7, HV) ^{YP}				-	-	-	+	-	+	+	94
YPT1				-	-	-	-	+	+	+	96
Ypt1-HV ^{S4}				-	-	-	-	+	+	+	96
Ypt1-L7 ^{S4}				-	-	-	-	+	+	+	85
Ypt1-(L7, HV) ^{S4}				-	-	-	-	-	-	-	-
Ypt1-EF ^{S4}				-	-	-	-	+	+	+	94
Ypt1-(EF, HV) ^{S4}				-	-	-	-	-	-	-	-
Ypt1-(EF, L7) ^{S4}				-	-	-	-	-	-	-	-
Ypt1-(EF, L7, HV) ^{S4}				-	-	-	-	-	-	-	-

pression of the temperature-sensitive *ypt1-1* mutation. In all cases when the chimaeras failed to suppress a deletion allele they also failed to suppress the temperature-sensitive allele. The levels of expression of Sec4 and Ypt1 from the integrated alleles were determined by quantitative western blot analysis to be within 10% of the wild-type expression level (data not shown). Dunn *et al.*⁸ found that an episomal *SEC4-L7^{YP}* construct could complement a *YPT1* deletion with moderate efficiency, we found, using an integrated construct, that complementation was inefficient, yielding microcolonies that were visible only after 4–6 days at 25 °C. As both the episomal and integrated constructs were tested in the same *ypt1Δ* strain, the most likely explanation for the discrepancy in growth rate is a difference in the levels of expression from the episomal and integrated alleles of the gene. We immunoprecipitated lysates derived from strains containing a number of the episomal-borne *SEC4*-based chimaeras (gift from B. Dunn and D. Botstein) and found that the Sec4 expression levels are 2–3 fold greater than the endogenous or integrated alleles of *SEC4*. Invertase assays were done as described²⁴. Numbers reflect the average of two independent isolates of each strain done in duplicate. The deviation between the two isolates was less than 4.0% in all cases.

FIG. 3 Immunofluorescence localization of *SEC4*, *YPT1*, and *SEC4-HV^{YF}* in *sec7-1* yeast cells. The left half of each panel is the fluorescein staining used to visualize Ypt1; the right half of each panel is Texas Red staining used to visualize Sec4: a-c, NY760 (*sec7-1*, *ura3-52*, *SEC4*, *YPT1*); NY885 (*sec7-1*, *ura3-52*, *sec4Δ::URA3*, *LEU2::Sec4-HV^{YF}*, *YPT1*). In a and d, cells are labelled with both primary antibodies; in b and e, they are labelled with anti-Ypt1 antibodies only and in c and f with anti-Sec4 antibodies only. For all panels, incubation was with both secondary antibodies. Scale bar, 10 μ m.

METHODS. Double immunofluorescence staining of yeast cells was done using affinity-purified rabbit anti-Ypt1 antibodies and cell culture supernatant from a mouse monoclonal cell line (C1.1) reactive against loop 7 region of Sec4. Rabbit anti-Ypt1 serum (gift from S. Ferro-Novick) was affinity-purified against SDS-gel-purified recombinant Ypt1²⁵ as described¹⁰. Mouse monoclonal antibodies were prepared against recombinant Sec4²⁶ and then epitope-mapped against various chimaeras. Monoclonal C1.1 was specific for Sec4 as it reacted with a single band in both immunoprecipitation and immunoblot analysis of whole cell lysates. Epitope mapping demonstrated that the 9-residue L7^{S4} domain was both necessary and sufficient for binding of this antibody on immunoblots. Yeast strains were prepared for immunofluorescence using cold methanol/acetone permeabilization¹². All samples were stained with a mixture of two affinity-purified secondary antibodies at a 1:100 dilution. Anti Ypt1 antibodies were detected with fluorescein (DTAF)-conjugated donkey anti-rabbit antibodies



Jackson Laboratories); anti-Sec4 antibodies were detected with Texas red-conjugated donkey anti-mouse antibodies (Jackson). Cells were viewed by confocal microscopy using a 63 $\times$ 1.4 NA objective on an Axiovert photomicroscope provided with a MRC600 confocal imaging system with an argon/krypton laser for illumination (Bio-Rad). Samples were simultaneously excited at 488 and 568 nm and the emission observed through a 560 DMP dichroic filter with a 522 DF35 filter for fluorescein emission and with a 585 EFLP filter for the Texas red emission. Separation of the emissions of the two fluorophores using this system is apparent from the lack of bleed through in single primary antibody staining (b, c, e, f).

not sufficient when it has to function at several transport stages.

It has been suggested that the Sec4/Ypt/rab family of proteins may play a central role in the intracellular targeting of vesicles to the appropriate acceptor membrane¹. The targeting model, in its simplest form, predicts that strains containing the *Sec4*-(L7, HV)^{YF} chimaera as the only copy of *YPT1* should form ER-to-Golgi vesicles with sufficient information to fuse with either Golgi or the plasma membrane. Figure 4a shows the

results of pulse/chase experiments with the vacuolar protein carboxypeptidase Y (CPY). If ER-to-Golgi vesicles are fusing with the plasma membrane, we would expect to see the appearance of the p1 (ER-modified) form of CPY in the external fraction. But both the control (*YPT1*)- and chimaera (*Sec4*-(L7, HV)^{YF})-containing strains showed normal transport of CPY to the vacuole without any evidence of missorting. Moreover, transport to the vacuole is more efficient in this strain

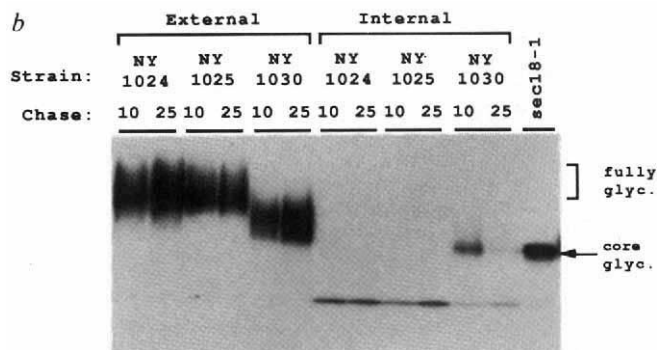
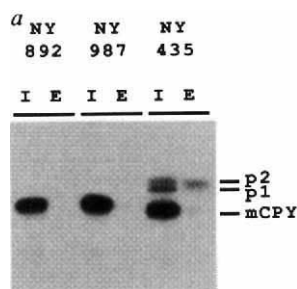


FIG. 4 Transport of CPY to the vacuole and secretion of invertase in a *Sec4*-(L7, HV)^{YF}/*ypt1Δ* strain is normal. a, Immunoprecipitation of carboxypeptidase Y. Strains NY892 (*YPT1*), NY987 (*ypt1Δ*, *Sec4*-(L7, HV)^{YF}), NY435 (*ypt1-1*) were labelled for 30 min with 200 μ Ci Tran³⁵S Label (ICN) and chased for 45 min with 0.5% methionine and cysteine. Internal and external fractions were prepared and immunoprecipitated with anti-CPY sera²⁷. Samples were electrophoresed on an 8% SDS-polyacrylamide gel. mCPY, mature carboxypeptidase Y; p1, core-glycosylated (ER-modified) pro-CPY; p2, Golgi-modified proCPY. Strains: NY892-*LEU2::YPT1*, *ypt1Δ::HIS3*, *his3-200*, *ura3-52*, NY987-*LEU2::Sec4*-(L7, HV)^{YF}, *ypt1Δ::HIS3*, *his3-200*, *ura3-52* NY435-*ypt1*, *ura3-52*. b, Immunoprecipitation of invertase. To facilitate the detection of invertase after immunoprecipitation, a 2 μ plasmid

containing the *SUC2* gene (pRB58) was transformed into NY892, NY987 and NY435 to yield NY1024 (*YPT1*), NY1025 (*ypt1Δ*, *sec4*-(L7, HV)^{YF}), and NY1030 (*ypt1-1*) respectively. Strains were derepressed by incubating in SD (0.67% yeast nitrogen base) with 0.1% glucose for 15 min and then labelled for 5 min and chased by adding methionine, cysteine and glucose to a final concentration of 0.5% each. Chase was for 10 or 25 min, as indicated. Internal and external (periplasmic) fractions were prepared and immunoprecipitated with anti-invertase serum. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel. As a marker for ER (core-glycosylated) form of invertase *sec18-1* strain (with pRB58) was preincubated and labelled at 37 $^{\circ}$ C and the internal fraction immunoprecipitated.

than in the *ypt1-1* strain, which shows an accumulation of transport intermediates and secretion of a small amount of Golgi-modified (p2) CPY even at the permissive temperature. Figure 4b shows the transport of invertase in the same three strains. Here, mistargeting of ER vesicles to the plasma membrane would result in secretion of the core-glycosylated form of invertase. The transport of invertase appears normal in both *YPT1-* and *Sec4-(L7, HV)^{YP}*-containing strains. Both show rapid secretion of fully glycosylated invertase, unlike *ypt1-1*, which shows transient accumulation of the ER form and secretion of an underglycosylated form of invertase¹. Taken together, these data suggest that Sec4 and Ypt1 do not, by themselves, act as molecular 'tags' to specify the acceptor compartment for a given vesicle. Rather, we believe, their primary function is as a switch to regulate the interaction of components required for the docking and fusion of transport vesicles with the appropriate membrane. □

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Specificity domains distinguish the Ras-related GTPases Ypt1 and Sec4

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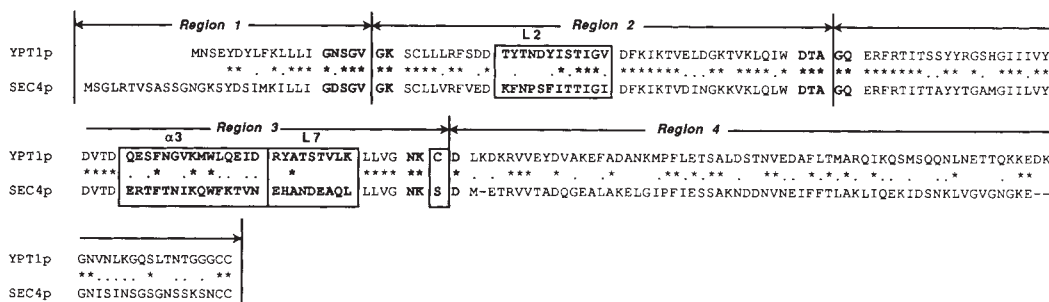
THE essential Ras-related GTPases^{1,2} Ypt1 and Sec4 act at distinct stages of the secretion pathway in the yeast *Saccharomyces cerevisiae*: Ypt1 is required for vesicular transport from the endoplasmic reticulum to the Golgi apparatus, whereas Sec4 is required for fusion of secretory vesicles to the plasma membrane³⁻⁶. Here we use chimaeras of the two proteins to identify a 9-residue segment of Ypt1 that, when substituted for the analogous segment of Sec4, allows the chimaera to perform the minimal functions of both proteins *in vivo*. This segment corresponds to loop L7 of the p21^{ras} crystal structure⁷. Substitution of a 24-residue Ypt1 segment,

including the residues just mentioned, together with 12 residues of Ypt1 corresponding to the 'effector region' of p21^{ras} (loop L2; refs 7, 8), transforms Sec4 into a fully functional Ypt1 protein without residual Sec4 function.

To determine which domain(s) within these two homologous proteins (which have ~50 per cent amino-acid identity) enables them to perform distinct cellular functions, we made precise reciprocal fusions between their coding sequences using a polymerase chain reaction (PCR) technique^{9,10} (Fig. 1). Three conserved motifs, here termed GxSGVGK, DTAGQ and NKxD (also designated G1, G3, and G4; ref. 11), were used as fusion joints. The ability of fusion proteins on low-copy plasmids to function as Ypt1 and/or Sec4 *in vivo* was tested by their ability to complement the conditional-lethal *ypt1-1*, or *sec4-8*, mutations or a *ypt1*-deletion allele; results of similar experiments are presented in an accompanying paper¹².

Reciprocal exchange of 5' or 3' non-coding sequences had no effect on the *in vivo* function of either gene (SY1, SY5, YS1, YS5; see Fig. 2) and the full-length genes complement only their cognate mutations (Fig. 2). The functional specificity must thus reside in the protein sequences themselves rather than in their regulation.

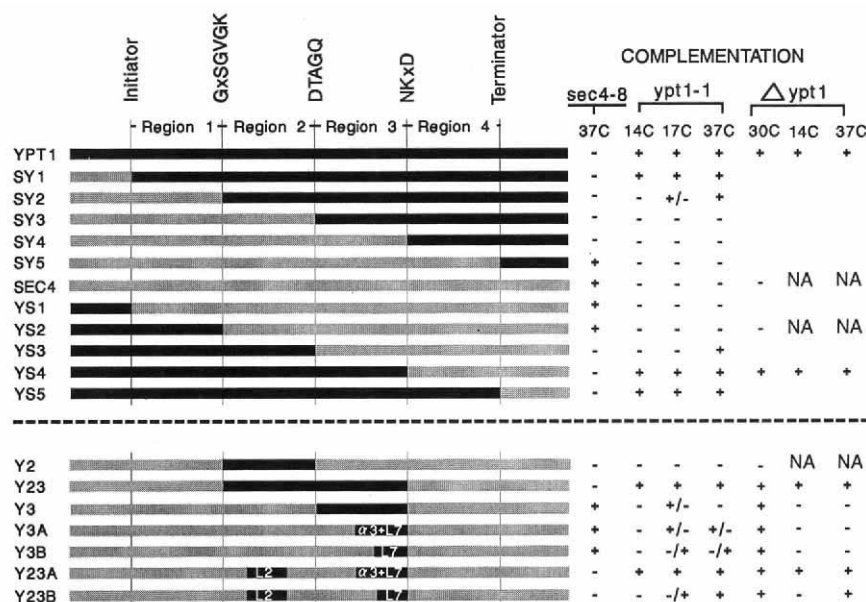
FIG. 1 The aligned amino-acid sequences of Ypt1 (ref. 22) and Sec4 (ref. 5) are shown. Asterisks indicate perfect amino-acid matches; dots indicate amino-acid similarities. The GxSGVGK, DTAGQ and NKxD motifs are shown in bold letters, and the fusion junctions within the motifs are shown as vertical lines. The amino-acid regions exchanged in chimaeric proteins Y3A, Y3B, Y23A and Y23B are shown in bold letters that are boxed and labelled; L2 refers to the 'effector region' (loop L2) region, α 3 refers to the helix α 3 region, and L7 to the loop L7 region, as described in the text. Note that the boxed residue within the NKxD motif is included in the L7 exchange region. Chimaeric protein Y3A consists of the entire



Sec4 amino-acid sequence except for the Ypt1 residues within the α 3 and L7 boxes; likewise, Y3B contains just the Ypt1 residues in box L7; Y23A contains just the Ypt1 residues shown in boxes L2, α 3 and L7; and Y23B contains just the Ypt1 residues in boxes L2 and L7.

FIG. 2 Diagrams of chimaeric genes and results of complementation assays. Solid black regions represent *YPT1* sequences and grey stippled regions represent *SEC4* sequences. The amino-acid sequences of the three conserved GTP-binding/hydrolysis motifs used as fusion junctions are shown at the top. For chimaeric proteins with fusion regions smaller than those between the conserved motifs, the exchanged regions are labelled L2, α 3 or L7, as described in Fig. 1. Relative growth rates are indicated as follows: a plus sign indicates robust growth on Ura drop-out plates²³ after 5–7 days at 14–17 °C or 2–3 days at 30–37 °C; +/- and -/+ represent increasingly less vigorous growth after these incubation times; and a minus sign indicates no growth after these incubation times. N/A, not applicable.

METHODS. We used a two-step PCR-based method⁹ to create fusion genes using a protocol similar to that in ref. 10. The final fusion products were cloned into the polylinker of the *URA3*-marked CEN plasmid pRS316 (ref. 24). Each construction was sequenced across the PCR junction. Plasmids were transformed using lithium acetate²⁵ into two haploid strains with conditional lethal alleles: DBY1803 (a *ypt1-1 ura3-52 his4-539 lys2-801*), which carries the cold- and heat-sensitive *ypt1-1* allele²⁶, and NY405 (a *sec4-8 ura3-52*), which carries the heat-sensitive *sec4-8* allele²⁷. The ability of each chimaeric protein to support growth at the relevant restrictive temperatures (14 °C, 17 °C and 37 °C for *ypt1-1* and 37 °C for *sec4-8*) was assayed. In addition, we assayed the ability of a subset of the fusion genes to complement the lethality of



a deletion of the *YPT1* gene. This was done by transforming the plasmids into a diploid strain heterozygous for a *ypt1* deletion marked with *HIS3* (NY 921; ref. 12). Recovery of His⁺ spore indicated that the chimaeric protein was able to rescue the spore; such spores were also tested for growth at 14 °C and 37 °C on YPD.

Reciprocal exchange of the small N-terminal region (to the GxSGVGK motif; Fig. 2, SY2 and YS2) had little or no effect. Moreover, almost the entire C-terminal half of Ypt1 (from NKxD to the C terminus; Fig. 2, YS4) can be swapped with that of Sec4 without loss of Ypt1 function *in vivo*. Any 'specificity domain' of Ypt1 must thus reside internally, between the GxSGVGK and the NKxD motifs. Although localization signals have been identified in the C-terminal regions of two mammalian members of the Ypt1/Sec4 family¹³, this need not contradict our result, because we assayed function rather than localization. The reciprocal hybrid (SY4) failed to complement any of the *ypt1* or *sec4* mutations; the protein may, however, be trivially defective.

To define the putative Ypt1 'specificity domain' more precisely, we substituted internal portions of Ypt1 into analogous regions of Sec4. A chimaeric protein containing a 104-residue internal region of Ypt1 and the N- and C-terminal regions of Sec4 (Fig. 2, Y23) functions as well as the wild-type Ypt1 at all

restrictive temperatures, including rescue of *ypt1*-deletion spores. Y23 actually functions better as Ypt1 than SY2, which has more Ypt1 sequences (Fig. 2), suggesting that the N- and C-terminal regions of Sec4 may interact.

Next we split the internal Ypt1 region of Y23 into two parts, using DTAGQ as a junction, thus yielding chimaeric proteins Y2 and Y3 (Fig. 2). Y2 contains 46 residues of Ypt1 sequence, including the 'effector loop' in small GTPases^{8,14,15}, but fails to complement any of these mutations (Fig. 2), allowing no conclusion to be drawn. Y3 contains 58 residues of Ypt1 between the DTAGQ and NKxD motifs, of which only 27 amino acids are different in the two proteins (Fig. 1). Surprisingly, Y3 can rescue *ypt1*-deletion spores, showing that it carries out the minimal functions of Ypt1, but it can complement *ypt1-1* only at the semi-permissive temperature of 17 °C (Fig. 2). Likewise, the *ypt1*-deletion spores rescued by Y3 (which had been germinated at 30 °C) are heat- and cold-sensitive. Y3 thus has a minimal Ypt1 function. Strikingly, it can also complement the *sec4-8*

FIG. 3 Glycosylation of invertase. Strain DBY1803 (*ypt1-1*; ref. 26) with or without plasmids carrying an intact *YPT1* gene or chimaeras Y3B, Y3A, Y23B or Y23A (same plasmids as for Fig. 2) were grown in liquid Ura drop-out medium²³ to mid-log phase at 30 °C (Y23A was grown at 25 °C), then shifted to low (0.1%) glucose conditions for 1.5 h. Whole cell extracts were prepared, then assayed by immunoblotting as described³, except that enhanced chemiluminescence (Amersham) was used for detection. Positions of molecular size ($M_r \times 10^{-3}$) markers (BioRad) are indicated on the left and of fully glycosylated and unglycosylated invertase on the right. The amount of Y23A extract loaded was inadvertently ~2–3 fold higher.

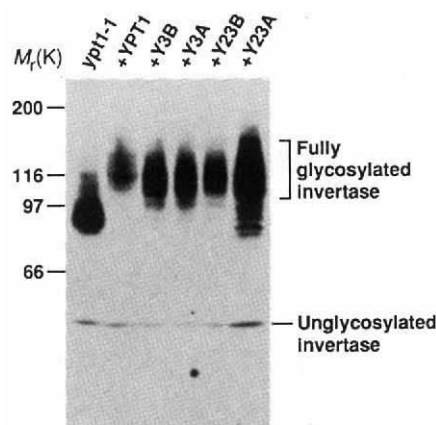
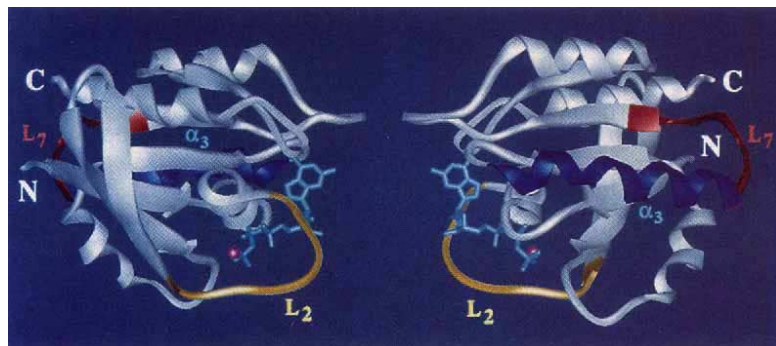


FIG. 4 Crystal structure of p21^{ras}. The molecule is shown in two views: the molecule on the right is rotated ~180° about the vertical axis relative to the molecule on the left. The regions of p21^{ras} corresponding to the loop L2, helix α_3 and loop L7 exchange regions (Fig. 1) are highlighted: L2 in yellow, α_3 in blue, and L7 in red (note that the residue corresponding to the Cys 123 of Ypt1 is not highlighted). The GTP is shown in cyan, and Mg²⁺ in magenta. The N and C termini are indicated. Figures were drawn with MidasPlus, developed by the Computer Graphics Laboratory at UCSF, using coordinates⁷ provided by A. Wittinghofer.



mutation completely at 37 °C (Fig. 2). This bifunctionality means that the specificity-bearing domain(s) of Ypt1 and Sec4 are not entirely overlapping, otherwise a swap would necessarily cause loss of one function while gaining the other. This result also shows that Y3 is stable at 37 °C; therefore its failure to function as Ypt1 at this temperature must reflect a specific feature of Ypt1 function.

To define the Ypt1 specificity domain more exactly, we created the chimaeric protein Y3A (Fig. 2), which contains the C-terminal 24 of the 58 Ypt1 residues in Y3. These 24 residues (21 of which differ in Ypt1 and Sec4; see Fig. 1) correspond to helix α_3 and loop L7 in p21^{ras} (Fig. 4; ref.7). We also created Y3B (Fig. 2), which contains only nine Ypt1 residues preceding the NKxD motif. These nine residues (8 of which differ in the two proteins; see Fig. 1) correspond to loop L7 in p21^{ras}. Both Y3A and Y3B also contain the Ypt1 Cys 123 within the NKxD motif, rather than the serine of Sec4. Both proteins are able to complement *ypt1* mutations (including the *ypt1* deletion) as well as the parent Y3, although Y3 and Y3A complement *ypt1-1* at 17 °C better than Y3B. Thus 9 Ypt1 residues (number 108, 109, 111–116, and 123) at most are required to confer minimal Ypt1 function on a related, but functionally distinct, GTPase. Both Y3A and Y3B still fully complement *sec4-8* (Fig. 2), indicating that they, like Y3, are bifunctional.

The results with Y23 indicated that we could confer full Ypt1 function upon Y3A and Y3B by restoring 12 residues of Ypt1 corresponding to the 'effector region' (loop L2) of p21^{ras} (eight residues of which differ in Ypt1 and Sec4), to create chimaeras Y23A and Y23B (Figs 1 and 2). Like Y23, Y23A complements the *ypt1-1* and *ypt1*-deletion mutants at all restrictive temperatures, but has lost the ability to complement *sec4-8*. Sec4 can thus be converted to a fully functional 'Ypt1' protein through the transfer of two short Ypt1 segments containing 36 residues (of which 29 differ in Ypt1 and Sec4). In contrast, Y23B (21 total residues with 17 differences), when compared to its parent Y3B, has gained Ypt1 function only at 37 °C, not at 14–17 °C. The loop L2 'effector region' thus seems to modulate the specificity of Ypt1 and Sec4 function at 37 °C, whereas the Ypt1

helix α_3 region influences Ypt1 function at 14–17 °C.

To assess how far our chimaeric proteins restored function, we examined glycosylation in *ypt1-1* strains with and without plasmids bearing Ypt1 or several of the chimaeras. All the functional chimaeras that we tested completely suppress the mis-glycosylation of invertase characteristic of *ypt1-1* (ref. 3; Fig. 3), showing that they correct at least one secretion defect in the *ypt1-1* mutant. As *ypt1-1* mis-glycosylates invertase even at a permissive temperature³, this criterion is quite rigorous.

Although at least four unlinked genes (*SLY* genes) have been identified that appear to bypass Ypt1 function^{16–19}, three do so only when highly expressed. Our complementing chimaeras differ in that they are highly homologous to Ypt1, almost certainly encode functional GTPases, and complement when expressed from a normal promoter at low copy number. (The *SEC4* gene, in contrast, cannot complement *ypt1* mutations even at high copy number⁵.) Most tellingly, the *SLY* genes are unable to suppress fully the misglycosylation of invertase^{16,17}. The chimaeras are therefore unlikely to bypass Ypt1 or Sec4 functions the way the *SLY* genes do.

The structures of Ypt1 and Sec4 are probably very similar to that of p21^{ras} (Fig. 4) as their sequences are homologous²⁰. Twenty-one of the 29 amino acids that distinguish Ypt1 functions from those of Sec4 lie in the region corresponding to loop L7 and helix α_3 in p21^{ras} (shown in Fig. 4 in red and blue, respectively). The putative 9-residue Ypt1 'specificity domain' (loop L7) is surface-accessible, and lies on the opposite side of the molecule from the remaining 8 of the 29 amino acids, which fall near the region corresponding to the GTP-binding pocket. (Fig. 4; loop L2, in yellow). It has been proposed²¹, on the basis of analogous studies with α -subunits of signalling GTPases, that the face corresponding to that surrounding loop L7 in p21^{ras} interacts with membranes or membrane-localized proteins. Results in the accompanying letter¹² make it unlikely that cellular localization alone is responsible for the different functions of the two proteins; for Ypt1, such specificity may well be achieved by interactions between these two regions and particular downstream ligands. □

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Structure of a major immunogenic site on foot-and-mouth disease virus

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ATTACHMENT of foot-and-mouth disease virus (FMDV) to its cellular receptor involves a long and highly antigenic loop containing the conserved sequence, Arg-Gly-Asp, a motif known to be a recognition element in many integrin-dependent cell adhesion processes¹⁻⁷. In our original crystal structure of FMDV the Arg-Gly-Asp-containing loop ('the loop'), located between β -strands G and H of capsid protein VP1, was disordered and hence essentially invisible. We previously surmised that its disorder is enhanced by a disulphide bond linking the base of the loop (Cys 134) to Cys 130 of VP2 (ref. 8). We report here the crystal structure of the virus in which this disulphide is reduced. Reduced virus retains infectivity and serological experiments suggest that some of the loop's internal structure is conserved⁸. But here its structure has become sufficiently ordered to allow us to describe an unambiguous conformation, which we relate to some key biological properties of the virus.

To break the disulphide bond, crystals of the virus⁹ were soaked in 10 mM dithiothreitol before data collection. Experimental details are presented in Table 1. In the refined structure

of the reduced virus, the loop folds snugly against the surface of the particle (Fig. 1), filling a minor surface depression and maintaining the relatively smooth external surface of the virus¹⁰. On breaking the disulphide bond, there is a large rearrangement of the residues leading into the loop itself; the C α -atom of residue Cys 134 of VP1 moves by some 12 Å and the released β E- α B loop of VP2 also moves significantly (the C α of residue 131 moves by over 2 Å), settling into a more ordered conformation. Beyond Cys 134 of VP1, the polypeptide chain adopts an extended conformation, sweeping out over the G-H loop of VP3 (which as a result refolds, with movements of up to 10 Å). The chain then passes back across VP2 towards the 3-fold axis of the icosahedron, residues 144-146 forming an additional strand of β -sheet overlying residues 80-82 in strand C of VP2. An open turn then leads into a 3_{10} helix which heads back

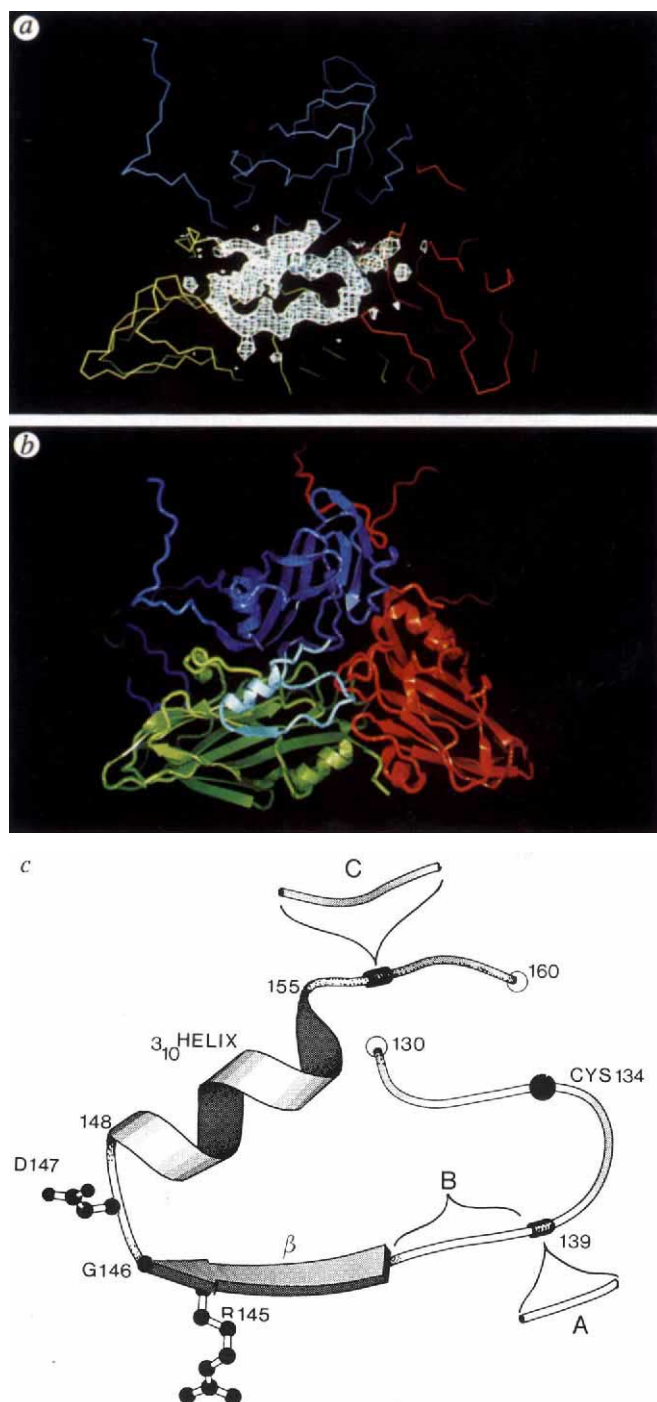


FIG. 1 The structure of the G-H loop of VP1 of FMDV. As in all picornaviruses, the capsid consists of three surface-oriented proteins, VP1-VP3, each with an eight-stranded β -barrel. The surface loops joining the β -strands are biologically important, none more so in FMDV than the G-H loop of VP1. **a**, Experimental evidence for the G-H loop structure. A difference electron density map calculated at 4 Å resolution with amplitudes $F_{\text{reduced}}^{\text{hobs}} - F_{\text{native}}^{\text{hobs}}$ and native phases (contoured at an arbitrary level and shown as a white mesh) showing clear continuous density defining the structure for the whole of the VP1 G-H loop. In the crystallographic analysis of the native virus, the electron density for the VP1 G-H loop was diffuse or absent between residue 134 (which participates in a disulphide bridge with residue 130 of VP2) and residue 156. The only other features in the difference map correspond to a refolding of the G-H loop of VP3, which interacts with the VP1 G-H loop in the reduced virus, a relaxation of the β E- α B loop of VP2 and the partial reduction of the less accessible disulphide bonds linking residue Cys 7 of VP3 around the 5-fold axis. Otherwise, the native and reduced viruses are almost indistinguishable. The polypeptide chain is coloured: VP1, blue; VP2, green; VP3, red. An electron density map with coefficients $2F_{\text{reduced}}^{\text{hobs}} - F_{\text{native}}^{\text{hobs}}$ and native phases showed clear side-chain density correlating exactly with the sequence for the loop residues and allowed a model to be built. The view here, and in the other figures, is from the outside of the capsid. **b**, A cartoon sketch³¹ illustrating the loop (pale blue) on the icosahedral asymmetric unit with the proteins colour coded as in **a**. **c**, A molscript³² ribbon diagram of the VP1 G-H loop with the side chains of the RGD sequence (residues 145-147) depicted using a 'ball and stick' representation. The position of Cys 134 is shown. Insertions and deletions in other serotypes of the virus are roughly located on the basis of sequence alignments: (A), up to three residues inserted in SAT serotypes; (B), up to four residues deleted in C, A and Asia serotypes; (C), up to four residues inserted in SAT serotypes.

towards the 5-fold axis and ends the loop (Fig. 1b). Models constructed using predictive^{3,11} and spectroscopic methods¹² for several serotypes of FMDV share few of these structural features. Although the G-H loop varies greatly both in sequence and length between the different serotypes of FMDV, the nature of these differences suggests that some general structural features of the loop are likely to be conserved (Fig. 1c). But only the O₁ viruses have both Cys residues required to form the disulphide bond between the VP1 G-H loop and VP2, and it will be interesting to see if structural analyses of other types of FMDV reveal an ordered structure for the loop.

Although the structure of the loop in the reduced virus is unambiguous, it remains one of the most mobile regions of the virus surface (as judged from the crystallographic *B*-factors). To assess the stability of the reduced form of the loop, we investigated the kinetics of reoxidation by doing a time-course analysis of the reformation of the disulphide. Two further three-dimensional structures were determined, 3 and 6 days after repeatedly washing the crystals in thiol-free medium to allow reoxidation (data not shown). The electron density maps indicate that the half-time for reformation *in vitro* at room temperature is of the order of 4 days. In purified virus the loop is predominantly disulphide-bonded (Fig. 2); thus this structure is not an artefact of crystallization. To determine at what stage in the life-cycle of the virus this disulphide bond is formed, we investigated the proportion of loops disulphide-bonded in virus at different times after release from infected cells (Fig. 2). These experiments show that, in freshly released virus, the reduced structure predominates and is therefore likely to be a biologically important species; however it seems that within 28 h the majority of the VP1-VP2 bonds have formed (a little more rapid than the timescale for reoxidation observed in the crystals). This agrees with the observation that the infectivity of the reduced virus is not significantly altered, demonstrating that it can still

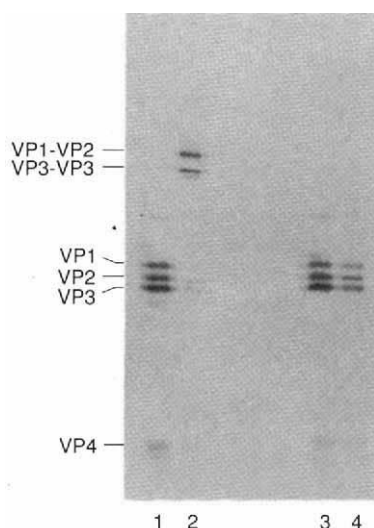


FIG. 2 The formation of disulphide bonds occurs after release of virus from the host cell. Virus-infected BHK-21 cells, labelled with ³⁵S-methionine and cysteine, were lysed in 0.5% Nonidet P-40, 0.04 M NaPO₄ pH 7.6, 0.1 M NaCl, and the viruses purified⁹ from these lysates were analysed by SDS-PAGE. The virus analysed in lanes 1 and 3 was carboxymethylated by including 50 mM iodoacetamide in the lysis solution, so preventing the formation of any disulphide bonds after release from the cell, whereas oxidation was allowed to proceed normally in the virus samples shown in lanes 2 and 4 (analysed 28 h post-release). To reveal disulphide-linked protein dimers, viruses were denatured in the absence of reducing agent (lanes 1, 2). Before loading on the gel, the nonreduced samples were also treated with 50 mM iodoacetamide for 2 h to prevent free thiols in the protein exchanging with disulphide bonds during electrophoresis (a reaction that can otherwise lead to serious underestimation of the amount of dimer). Lanes 3, 4 show controls denatured in the presence of 0.4% (v/v) mercaptoethanol.

TABLE 1 Summary of crystallographic data collection and analysis

Data set	Number of filmpacks processed	Completeness to 2.6 Å (%)	<i>R</i> (I) (%) (all data)	<i>R</i> _c (%)	Stereochemistry
Native	88	84.6	14.1%	18.6 (6.0–2.8 Å)	r.m.s. Δ _{bonds} : 0.025 Å r.m.s. Δ _{angles} : 4.2° r.m.s. Δ _{B_{bonds}} : 3.3 Å ² r.m.s. Δ _{B_{angles}} : 5.3 Å ²
Reduced	14	53.0	16.2%	20.8 (52.0–2.6 Å)	r.m.s. Δ _{bonds} : 0.023 Å r.m.s. Δ _{angles} : 3.7° r.m.s. Δ _{B_{bonds}} : 7.7 Å ² r.m.s. Δ _{B_{angles}} : 9.6 Å ²

r.m.s. Δ _{bonds} refers to the root mean square deviation from ideal covalent bond lengths. r.m.s. Δ _{angles} refers to the root mean square deviation from ideal covalent bond angles. r.m.s. Δ _{B_{bonds}} refers to the root mean square difference in isotropic *B*-value between covalently bonded atoms. Δ _{B_{angles}} refers to the corresponding difference between covalently bonded next nearest neighbours.

$$R(I) = \frac{\sum_n \sum_i (|I_n - I_{hi}|)}{\sum_n \sum_i I_{hi}} \times 100$$

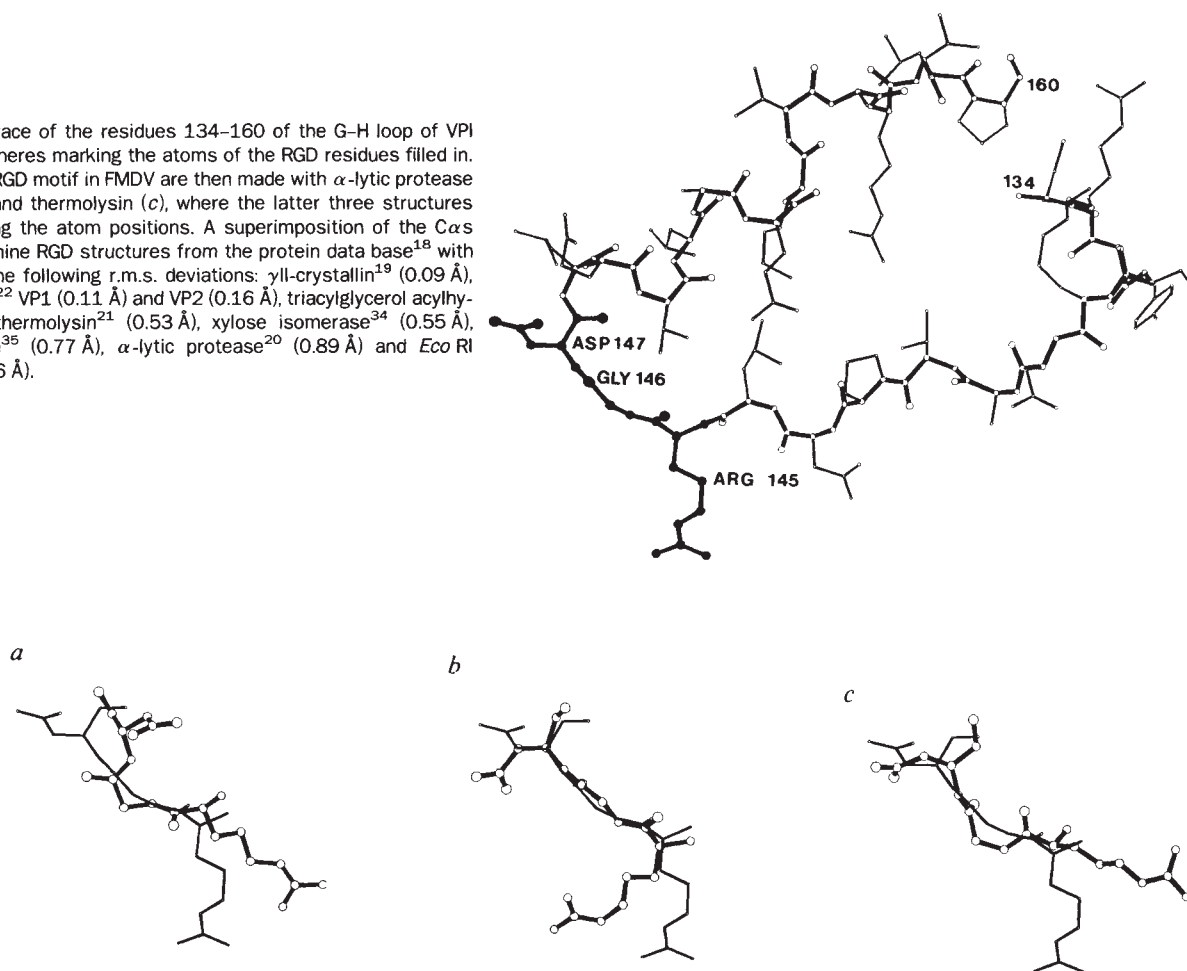
$$R_c = \frac{\sum_n (|F_{nobs}| - |F_{ncalc}|)}{\sum_n |F_{nobs}|} \times 100$$

I_n is the weighted mean measured intensity of the observations I_{hi} . F_{nobs} is the observed structure amplitude of reflection h . F_{ncalc} is the structure of reflection h calculated from the appropriate model. Crystals of the virus⁹ were soaked for some 2 h in crystallization mother liquor to which 10 mM dithiothreitol had been added and quickly mounted in sealed quartz capillaries to minimize reoxidation. Data were collected at the SERC Daresbury synchrotron radiation source station 9.6, $\lambda = 0.89$ Å on 0.5° oscillation photographs. The size of the crystals soaked (up to 0.4 mm in the longest dimension) generally exceeded those used in the determination of the native structure¹⁰, and so diffraction data were collected to a resolution of 2.6 Å. The mean fractional isomorphous difference between the native and reduced data was 20.7% on all data. Extra native data were also collected and processed, and compared with those used in the original structure determination as a control; this showed that the new native structure was indistinguishable from the old. The protocols for data collection and processing were as previously described²⁸ except that in this case partially recorded reflections on films from adjacent oscillation ranges were summed (two film packs were obtained from several crystals), and those reflections more than 80% recorded on a single film were scaled to 100%. The virus structure was refined against the data collected for the reduced virus with the package X-PLOR, version 3.0²⁹. No account was taken of the scattering from bound waters; however, a bulk solvent correction was applied, hence the good agreement of the model over a wider resolution range than the native. A tight solvent mask was defined, with a sufficiently high average value for the solvent density to account for bulk solvent and RNA. All data including those recorded as negative intensities were retained throughout the processing and included in the refinement³⁰. Exact 5-fold non-crystallographic symmetry constraints were included throughout the refinement of atomic positions and individual *B*-values. Using the full resolution range of the data and a suitably weighted bulk solvent mask it was possible to determine the relative occupancy of the G-H loops of VP1 and VP3 in the reduced structure (0.85 ± 0.15), giving us greater confidence in the *B*-factors of these regions (methods will be published elsewhere). Coordinates for the native and reduced virus structures have been deposited with the protein data bank¹⁸.

bind to the cellular receptor (data not shown and ref. 8), although the kinetics of cell binding in the reduced and oxidized states have not been compared.

FMDV was the first virus for which an integrin was implicated in cell attachment^{4–6,13}, although the exact identity of its receptor remains unknown. Recent results indicate that some other picornaviruses use receptors of this family^{14,15}. Unfortunately there are as yet no direct structural results on integrin–ligand interactions. The residues Arg-Gly-Asp (RGD) are of particular importance in integrin recognition^{16,17}, and in FMDV this sequence (residues 145–147) is highly conserved and centrally placed in the G-H loop. We have compared the conformation of this triplet in FMDV with nine other occurrences of the motif in the Protein Data Bank¹⁸ (Fig. 3). Three of these are reported to have been assayed for integrin binding activity *in vitro*¹⁷. The RGD motif in FMDV is very similar to that of γ II-crystallin¹⁹, the only one to bind an integrin actively, and differs from those of the inactive proteins^{20,21} (for details see Fig. 3). Of the other RGD motifs in the Protein Data Bank, the one located in an external loop of the coat protein VP1 of human rhinovirus 1A (ref. 22) (whose receptor is unknown) is the most similar in structure to the motif in FMDV, ranking alongside γ II-crystallin. In both γ II-crystallin and FMDV, the acidic and basic side

FIG. 3 An all-atom trace of the residues 134–160 of the G–H loop of VPI is shown with the spheres marking the atoms of the RGD residues filled in. Comparisons of the RGD motif in FMDV are then made with α -lytic protease (a), γ -crystallin (b) and thermolysin (c), where the latter three structures have spheres defining the atom positions. A superimposition of the C α s only for each of the nine RGD structures from the protein data base¹⁸ with that in FMDV gave the following r.m.s. deviations: γ II-crystallin¹⁹ (0.09 Å), human rhinovirus 1A²² VP1 (0.11 Å) and VP2 (0.16 Å), triacylglycerol acylhydrolase³³ (0.53 Å), thermolysin²¹ (0.53 Å), xylose isomerase³⁴ (0.55 Å), tryptophan synthase³⁵ (0.77 Å), α -lytic protease²⁰ (0.89 Å) and *Eco* RI endonuclease³⁶ (1.16 Å).



chains of this tripeptide are in an 'open' conformation, well separated from each other. Nuclear magnetic resonance (NMR) studies on peptide inhibitors of integrins suggest that such a conformation enhances their potency^{23,24}. Recently, structural information has become available for the disintegrins, a family of polypeptide integrin inhibitors^{25,26}. A common feature of these is the presence of an RGD motif in a mobile loop, a situation similar to that in FMDV. This mobility has made it difficult to determine the structure of the RGD region in the disintegrins. We are fortunate that in FMDV the RGD triplet is stabilized by the involvement of residues 145–146 in β -sheet interactions (the mean *B*-factors for the backbone atoms of the RGD being lower than the average for the loop). A further active RGD has recently been visualized, that of the fibronectin type III domain from tenascin²⁷. This is also found to be in an open conformation at the turn of an extended loop although it is not yet possible to make a detailed comparison.

The VPI G–H loop seems to act as a self-contained unit, probably with some internal flexibility, hinged to the rest of the structure. Because the loop structure alters after release of virus from cells, time-dependent antigenic variation seems to be occurring in this virus. The structure for this loop suggests that part of the specificity of integrin–ligand interaction derives from the particular conformation of the polypeptide backbone of the RGD residues of the ligand existing on a mobile exposed loop. □

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The *Caenorhabditis elegans* genome sequencing project: first steps in automation

Andy Watson, Nicky Smaldon, Roger Lucke and Trevor Hawkins

Novel biochemical processes using magnetic particles have been automated to provide a robotic system to perform processes for large-scale shotgun sequencing.

THE development of automated devices to facilitate high-throughput DNA sequencing is essential to the future success of the large-scale genome projects. Before designing any robotic system it is important to have an appreciation of the scale of operation and the difficulties that this might impose. For example, the completion of the 100-Mb *Caenorhabditis elegans* genome will require in excess of 10^6 DNA templates, all of which will need to be sequenced, identified and tracked at all times from plaque picking to entry in the database.

In an effort to increase the throughput of the *C. elegans* genome project¹, we have simultaneously designed a robotic system and developed several biochemical procedures to begin the automation of the shotgun sequencing process.

The approach adopted to sequence the *C. elegans* genome has been to create M13 subclone libraries from overlapping cosmids. For each cosmid 800–900 random M13 clones are sequenced with the M13-21 universal primer to provide the initial shotgun coverage of 95–98 per cent of the final contig length. This sequencing strategy has enabled us to identify several procedures that would benefit from automation. The first step is a plaque-picking procedure followed by the growth of the M13 clone in culture. The next step is purification of the DNA template and then the sequencing of each M13 clone before being loaded on to the DNA sequencer (Model 373A, Applied Biosystems, Foster City, California).

Ideally, the sequencing process should be continuous and integrated. It therefore seemed logical that our robotic device should provide a complete system. Such a system could take samples through the whole process, from plaque picking to sequenced DNA ready for gel electrophoresis, thereby avoiding the creation of new bottlenecks.

Robotic system

The system (Fig. 1a and 1b) consists of an xyz robot (LR3-3, Berger Lahr UK Ltd, Slough, UK) with custom tools, and a working area comprising several fixtures as described below. The system is controlled by a PC-compatible computer with control and scheduling software written in TURBO C.

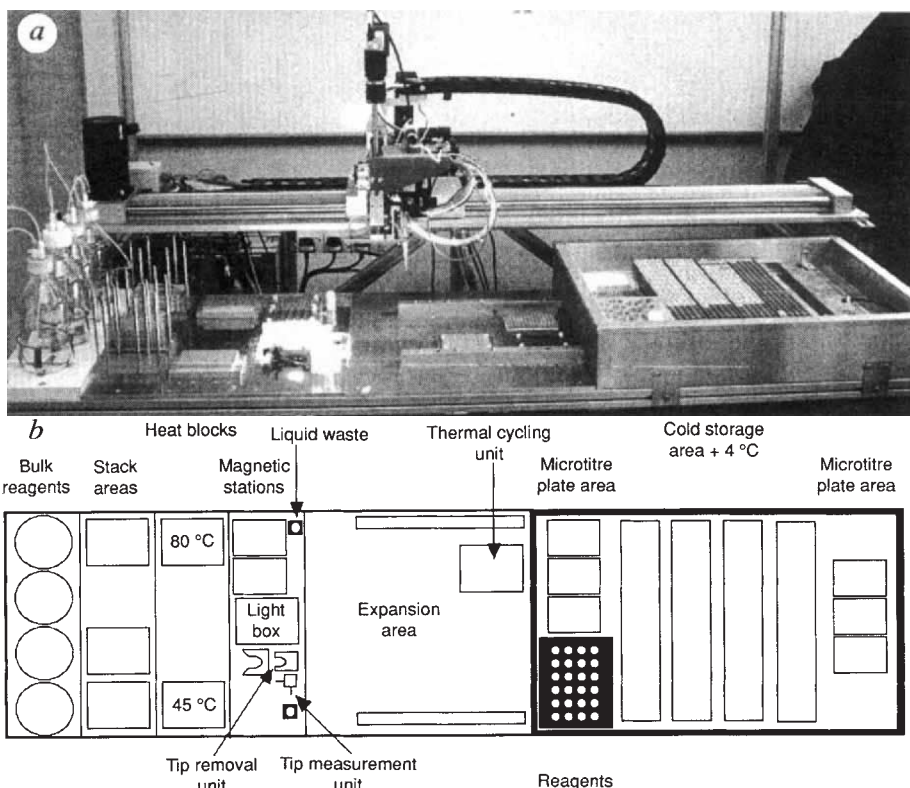


FIG. 1a, MRC mk II robotic system and b, a schematic view of the mk II working area as shown above. The robot arm has access to several fixtures such as stack areas, heat blocks and cooled reagents, which are used during normal operation. The working area has several expansion areas that will allow for future developments and modifications. The vibratory bowl feeder (see text) is not shown.

ware written in TURBO C.

A pipetting tool is attached to a motorized syringe (Compudil SR, Hook and Tucker Instruments Ltd, Croydon, UK), which allows volumes from 0.25 μ l to 1 ml to be pipetted in steps of 0.25 μ l. Yellow tips can optionally be picked up for sterile pipetting. Plates can be lifted and released by applying vacuum-to-vacuum pads. A video camera (TM6-CN, Pulnix Europe Ltd, Hampshire, UK) is also attached.

In the working area are four plate-stacking fixtures, each capable of storing ten Falcon microtitre plates (Falcon 3911 MicroTest assay plate, Becton Dickinson, California) or eight standard, round 9-cm Petri dishes in holders. Heat blocks (DB1M, Techne Ltd, Duxford, UK) at 80 °C and 45 °C are for

incubation. A 96-way magnetic station and strip magnet constructed using neodymium magnets (BM 89.306 A, Bakker Madava BV, 5691 MX Son, Netherlands) allow separation of magnetic particles. A light box station provides uniform illumination for the imaging of plaque plates. A fixture to hold one yellow tip and tip removal unit are provided along with a tip measurement device (yellow tip measurement is possible to a resolution of 0.075 mm). A thermal cycling block (Techne PHC-3) is used for cycle sequencing. A refrigerated area (4 °C) stores reagents, up to three 384-well microtitre plates (Genetix Ltd, Christchurch, UK), and up to 600 2-ml Eppendorf tubes in trays of 120. There are further unused areas available to allow for expansion.

A vibratory bowl feeder (Riley Automation, Derby, UK) feeds correctly orientated tips on to the tip pick-up station. Up to 3,000 sterile tips (stock number 70.760.002, Starstedt Ltd, Leicester, UK) can be sorted and fed. Also, bulk reagents can be fed directly to the tip, selected using a 5-port valve (Omnifit Ltd, Cambridge, UK).

Plaque picking and growth

Petri dishes in holders are placed in the storage racks by the user. The robot then moves a dish to the light-box station and finds the plaques using image-processing techniques². A sterile yellow tip is picked up and measured at the measuring station, providing a correction factor for nonuniform tips³. This tip is then used to pick each M13 plaque into a 2-ml Eppendorf tube. The tubes are then incubated at 36 °C for 5–6 h, shaking at 300 r.p.m. After growth they are centrifuged at 12,000g to clear the supernatant and are placed back on to the robot. This growth and centrifugation stage is the only user intervention required, although prospects exist for carrying out these steps on the machine by including an incubated growth area, then clearing the supernatant by filtering.

One advantage of placing the centrifuged Eppendorf tubes on the robot workspace is to allow the pelleted host cells to be pipetted by the robot to create glycerol stocks in microtitre plates, which are stored at 170 °C for subsequent use during the finishing stage.

M13 DNA purification

We have developed a method that uses streptavidin-coated magnetic particles (Promega Corp., Madison, Wisconsin) linked to a probe that is complementary to the target M13 template⁴. The procedure was designed to operate in standard microtitre plates using one well per sample. The DNA yield from this configuration is 600–800 ng DNA, which is sufficient for one thermally-cycled DNA sequencing reaction using *Taq* polymerase⁵. As this procedure does not use hazardous chemicals such as phenol, and is not dependent upon exact reaction volumes or temperatures, it is ideal for automation. This magnetic purification procedure, when performed manually, would allow one technician to purify six times the number of templates per day than if using traditional polyethylene glycol (PEG)/phenol methods⁶.

The automated production of M13 templates for the Cambridge *C.elegans* project has been in operation for 8 months. To date, over 40,000 templates have been purified and consequently sequenced. To verify the quantity of DNA being produced, several samples have been co-purified manually and robotically with a proportion of the DNA produced loaded onto an agarose gel. The photograph of the gel in fig. 2 shows three clones that were co-purified in this way showing that identical DNA yields were obtained. We have not observed any



FIG. 2 Relative yield between the M13 magnetic purification system performed by the robot and by hand. "M" denotes the lambda *Hind*III molecular weight marker (100 ng). Two microlitres out of a total of 20 µl of each sample were loaded. This demonstrates that the robotic templates are of similar yield and quality to those produced by hand.

differences in the subsequent DNA sequencing results between samples prepared manually and robotically in terms of DNA quality.

A further test was used to compare the data quality obtained from this robotic method as compared to the previous PEG/phenol procedure. To do this, two completed cosmid projects were chosen: ZK512, which had been sequenced using templates prepared by hand using the PEG/phenol method, and K11H3, which had been sequenced using robotically prepared templates. From each cosmid 80 random reads were chosen and the original ABI base calling, from the cloning site to base 300, compared to the final edited consensus sequence. From this, a mean error rate could be established. For the cosmid ZK512 the error rate was 1.1 per cent, whereas the robotically sequenced cosmid showed an error rate of 1.4 per cent. Errors were made up from "Ns" or no calls plus errors that were composed from miscalls, over calls and under calls. The difference noted here is not significant and shows that the quality of these data from the two approaches is similar despite the dramatic difference in the time required to prepare the two template sets.

DNA sequencing

The final stage is sequencing the template DNA. We have been using the robotic system to set up DNA sequencing reactions in standard Techne 96-well polycarbonate plates. For ABI sequencing reaction chemistries, once the samples have been cycled they are removed from under oil, pooled and placed in tubes with ethanol/salt solution. The samples are then removed from the machine to complete the precipitation process before loading. To date, two cosmids (approximately 1,800 clones) have been sequenced in this way; template DNA being prepared on the robot followed by DNA sequencing performed in Techne polycarbonate plates.

Summary

Although other molecular biology robots exist (Catalyst, ABI; ROB, Pharmacia, Uppsala, Sweden; Biomek 1000, Beckman, Fullerton, California), we found they had limited capacity, low throughput and were not flexible enough to allow the user to implement future improvements or changes. In most cases, these systems address only the mechanization of certain aspects of the sequencing process and require almost constant intervention by the user. The system described here can process up to 600 samples per day, automating all the pipetting operations required for thermal-cycle sequencing and M13 template preparations. Future prospects include the growth of phage on the machine followed by clearing of the supernatant by filtering. This would then make it possible to perform the whole sequencing process from plaque identification and picking to the creation of raw sequence data.

Although our goal has been to automate the initial shotgun stage, this has inevitably led to bottlenecks in gel electrophoresis and in the completion of finished sequence. Recent descriptions of capillary electrophoresis⁷ and long slab-gel systems^{8,9} will tackle these problems by allowing increased throughput and longer reads. Clearly, our machine could be used to automate the sequencing chemistries and approaches used in the completion stages, and new chemistries are being developed for this task. □

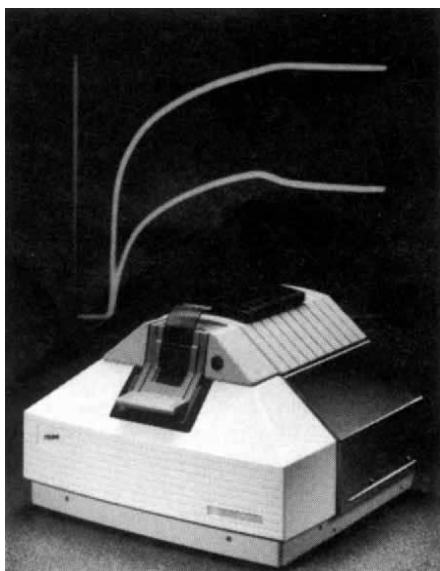
Andy Watson and Nicky Smaldon are at the Sanger Centre, Hinxton Park, Hinxton, Cambridge CB10 1RQ, UK, Roger Lucke is at the Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK, and Trevor Hawkins is at Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. We would like to thank Andrew Smith for providing K11H3 cosmid sequence data, John Sulston for specific comments and encouragements, and the members of the *C.elegans* mapping and sequencing group in Cambridge, UK and St Louis, Missouri, USA. This work has been supported by grants from MRC-Human Genome Mapping Project and the National Institutes of Health Genome Centre. For more information, fill in reader service number 100.

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DNA developments to watch

Molecular research tools, gadgets and gismos featured this week include a new instrument for the real-time analysis of molecular interactions, an antibody for cystic fibrosis research and a hand-held DNA sequence recorder.

MOLECULAR interactions, including the analysis of biomolecular recognition, concentration, kinetics and binding, can be monitored in real time with the new IAsys system from Fisons Applied Sensor Technology (*Reader Service No. 101*). Fisons says that the instrument should be of interest to researchers in the fields of immunology, molecular biology, cell biology and biochemistry and should aid studies of structure-function relationships or the design of biomolecules with specifically altered activities. Studies of enzymic interactions in AIDS research, cell adhesion in cancer studies and novel drug development are some



IAsys — for real-time interaction analysis.

examples. IAsys is based on optical evanescent wave technology, which has been designed to combine the simplicity of surface plasmon resonance with the enhanced sensitivity of wave guiding techniques. The instrument measures the changes in refractive index and thickness occurring in the evanescent field when one partner of an interacting pair transfers from solution and binds to the other partner immobilized on the surface of the sensor. Affinity profiles available with IAsys — without the need for labels or sample purification — enable studies of molecular recognition, concentration, cooperativity, kinetics and multimolecular interaction mapping for a wide range of ligands and binding partners. A re-insertable sample cell, which incorporates its own optics, is designed to make IAsys easy to use in addition to reducing maintenance costs. The cell allows rapid changes in chemistries to be studied, off-

instrument procedures for ligand attachment and multiuser flexibility. An optimized regeneration protocol allows repeated use of the cell for most applications.

Genzyme is distributing the new **monoclonal mouse anti-human CFTR (C-terminus specific) antibody**, which should be of interest to those biochemists, cell biologists and molecular biologists studying biochemical and genetic mechanisms of cystic fibrosis, working on new therapies or performing clinical trials on new drugs and treatments for this disease (*Reader Service No. 102*). New Brunswick Scientific, the UK distributor for Genzyme, says that the antibody is comparatively easy to use for immunoprecipitation, it can be used in a sandwich ELISA paired with R-terminus specific CFTR antibody (also available from Genzyme), and it works well in western blots to detect CFTR protein from transfected cells expressing high levels of CFTR.

MetaPhor **agarose** is a new gel from FMC BioProducts that forms strong gels which finely resolve polymerase chain reaction products and DNA fragments similarly to 5.5 per cent to 8 per cent polyacrylamide gels, detecting a size difference of 2 per cent in the range of 200 to 800 base pairs (*Reader Service No. 103*). The agarose, which is designed to combine the convenience of agarose with the resolution power of polyacrylamide, is compatible with most electrophoretic and blotting equipment.

■ Devices and gadgets

Sequenceboy is the new portable, **automated DNA sequence recorder** from Northumbria Biologicals that is intended to simplify nucleic acid sequence recording and editing (*Reader Service No. 104*). No larger than a pocket calculator, Sequenceboy can be operated in two ways: bases are identified by eye and entered manually using either the keypad on the console or the Reader Mouse keys. Sequences can be read anywhere at any time and transferred later to laboratory computers for analysis. Sequences are entered and can then be proof-read simply by re-entering these data. Discrepancies are signalled by a warning tone and a request is made for corrections to be made. As a portable unit, Sequenceboy can be used with battery power or connected to the mains. After recording and editing, sequences are stored in one of 15 independent memories of 1,000 bases each and interfaces are available to transfer stored data to either IBM-compatible or Apple Macintosh computer models. The



Sequenceboy — a portable, automated DNA sequence recorder.

A, C, G and T keys can be reallocated and matching acoustic signals turned on or off. An illuminated LCD shows the sequence in blocks of ten bases, together with the cursor position, total number of stored bases and memory bank number. The menu text can be displayed in English, German, French or Spanish and a password ensures security.

The new version of the Lynx **densitometer** from Applied Imaging is designed to be over five times faster than the earlier model (*Reader Service No. 105*). In addition, the Lynx now includes as part of its one-dimensional package, the ability to capture and analyse gels stained with ethidium bromide directly. Lynx also offers a gel documentation capability along with the system so that at any point an inexpensive video print can be generated. In addition to the direct analysis of gels stained with ethidium bromide, Lynx can also analyse other types of gels or blots. Additional software packages are available for DNA sequencing and two-dimensional gel analyses. Applications that are supported by Lynx include polymerase chain reaction quantitation, Southern, northern and western blots, dot and slot blots, fluorescent gels, one-dimensional gel analysis, smile correction, counting, gel documentation, DNA sequence analysis, and two-dimensional gel analysis.

The new UNO-Thermoblock from Biometra is a compact **thermocycler** designed for research, routine and diagnostic

applications (*Reader Service No. 106*). The new model, which is a smaller version of the TRIO-Thermoblock, can accommodate up to 40 samples (Eppendorf Safe Lock 0.5 ml). A simple modular exchange allows the instrument to work with microtitreplates, opening up applications in diagnostics, amplification screening and sample preparation for sequencing reactions. Using the Peltier technique the instrument is independent from ambient temperature and water bath. Fast and precise temperature profiles can be driven in the temperature range from +4 °C up to 100 °C. Heating and cooling rates (1.5 °C s⁻¹ and 1.3 °C s⁻¹, respectively) can be reduced via ramping functions down to 0.01 °C. Biometra says that this enables the instrument to perform rapid amplifications with low heating rates and precisely-controlled low cooling rates for sequencing reactions. All programs and temperature profiles can be documented on a computer printer.

■ Plasmid preparation methods

Amersham has extended its range of FMP miniprep systems with the introduction of FMP for **plasmid minipreps** (*Reader Service No. 107*). Based on fast magnetic purification, a technique for the entrapment and separation of precipitates using magnetic rather than centrifugal force, the new system has been developed specifically for the rapid isolation



Purification of plasmid DNA sequencing templates without centrifugation.

of plasmid DNA templates for use in dideoxy sequencing. FMP can be used to process multiple samples of plasmid DNA from small-scale bacterial cultures. The method requires no organic solvents and no centrifugation. Amersham says that high-quality template is generated for sequencing using either radioactive or fluorescent techniques. The technology is based on uncoated, nonderivatized magnetic particles. These small, dispersed superparamagnetic FMP particles act as a solid phase around which the precipitate can form. Application of a magnetic field causes the particles to aggregate and migrate to the pole of the magnet, taking the precipitate with them. Only when the magnet is removed do the particles disperse, enabling the trapped precipitate to be recovered in a suitable solvent. Two pack sizes are available sufficient for 50 or 100 preparations. A

FMP magnetic separator is available separately.

Even the most rigorous plasmid or cosmid preparation methods often fail to remove trace amounts of bacterial genomic DNA. Such contamination may result in serious difficulties in the characterization, subcloning or sequencing of cloned DNA, especially if the cloning vector is a low-copy-number plasmid or cosmid. Plasmid-Safe DNase from Epicentre Technologies is designed to provide researchers with a fast and easy method of selectively and completely **removing contaminating bacterial DNA in preparations of plasmid or cosmid DNA** (*Reader Service No. 108*). At neutral pH, the enzyme digests linear double-stranded (ds) DNA and both closed-circular and linear single-stranded DNA, but has no activity on nicked or closed-circular ds DNA. The enzyme will remove contaminating bacterial DNA in any plasmid preparation, effectively eliminating the possibility of subcloning or sequencing contaminant DNA, Epicentre says. Plasmids or cosmids treated with Plasmid-Safe DNase will also yield the maximum possible transformation efficiencies. The enzyme can be conveniently inactivated by incubation at 70 °C for 15 min.

■ Separation techniques

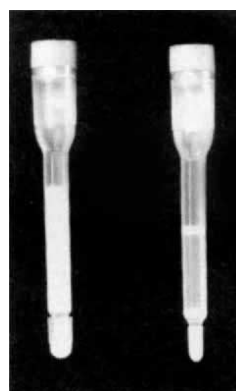
Millipore has announced a new high-resolution approach to separate proteins from very small amounts of sample and a way of collecting them for post-run compositional analysis. This allows protein chemists to **separate proteins and collect them directly onto a porous polymer membrane** (*Reader Service No. 109*). The proteins on the membrane can then be analysed directly with techniques such as western, northern and Southern blots, as well as direct amino acid analysis and protein sequencing. The proteins are separated and collected using



Waters Quanta 4000E capillary electrophoresis system with membrane fraction collector.

the Waters Quanta 4000E capillary electrophoresis system with the company's Waters Membrane Fraction Collector. It features direct collection of samples onto a porous membrane support with 100 per cent recovery, no dilution, while maintaining 100 per cent spatial resolution, Millipore claims. Design features of the Quanta 4000E CE system include a 185-nm UV detector that allows chemists to detect impurities at levels of 0.1 per cent, large buffer reservoirs to automate methods development, Accupure Z-1 Methyl Reagent that prevents proteins from binding to the wall of the capillary, and low-volume sample injection that offers an injection range of 5–500 µl.

Integrated Separation Systems has introduced a new line of prepacked **gel filtration columns**, which are suitable for



Desalting columns from ISS.

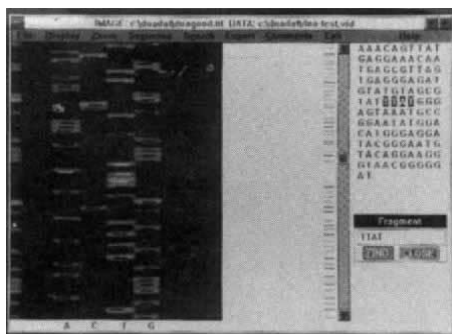
the purification or desalting of samples with volumes up to 0.5 ml (*Reader Service No. 110*). Available prepacked with either GF-25 resin (for oligos and proteins) or GF-50 resin (for nick translations), each autoclaved column is equilibrated in DNase- and RNase-free water. The top and the bottom of

the bed are fitted with porous polyethylene frits to protect the gel during storage and use. The company says that DNA or protein samples can be processed in two to four minutes.

■ Software selection

To complement its recently launched GelBase software package, Ultra Violet Products has introduced GelSeq, a new **DNA analysis program** designed to improve the accuracy of DNA sequencing from autoradiographs and gel images (*Reader Service No. 111*). It can be used with the company's System 5000 gel documentation system or, alternatively, as a standalone package. A film or gel is scanned by a track-mounted 256 grey-level optical scanner and the image is loaded into the software. As scanning takes place, a high-resolution, magnified image of the sequenced gel tracks is displayed on the PC's monitor. With the aid of contrast control, the screen's highly magnified image allows closely packed bands to be examined in detail. The image can be stored on disk for later analysis or imported as a TIF file from the gel documentation system's ImageStore 5000 unit. Once a scanned image is on display, base lanes can be identified (adenosine, guanine, cytosine and thymidine). By clicking the mouse on a band,

sequence data are displayed next to the image and, even if the bands are fused or the lanes distorted, predictor bars allow the next band to be selected with ease, UVP says. If required, contrast and zoom adjustments can be used to enhance faint bands or to magnify selected regions, and after the sequence has been stored, comparison with other



UVP's GelSeq DNA analysis software.

experimental data is designed to be quick and simple. Operating in the Windows 3.1 environment, GelSeq runs on most IBM PC or compatible computers. A minimum of 4 MB RAM, a mouse and a VGA monitor are required. An optional 256 grey level hand-held scanner is also available.

Primer prediction and selection capabilities for the polymerase chain reaction and sequencing applications have been added to the new 4.0 version of MacVector **sequence analysis software** from International Biotechnologies (*Reader Service No. 112*). Version 4.0 is also available with AssemblyLIGN, a sequence assembly and editing program. Researchers wishing to amplify a DNA fragment may use MacVector's new analysis to search for appropriate primer pairs according to product size or the region in which they occur. The program calculates melting temperature using the 'nearest neighbour' method. It also lists the numbers of primers and primer pairs it has considered, accepted and rejected. And, it lists the reasons for rejection so that researchers can adjust their parameters to expand or contract the list of primers accordingly. Acceptable pairs are listed with their sequence, length, T_m and G + C content along with the location, length, T_m and G + C content of amplified product. An optimum annealing temperature for the reaction is also calculated. Researchers can view recommended primer pairs in a compact graphical format that makes it easy to compare primer locations and product lengths. The new version also allows users to screen sequences for DNA sequencing primers or hybridization probes.

Detection systems

The Cool Glow **DNA sequence detection system** from Betagen, a division of Intelli-Genetics, is a complete reagent kit for NATURE · VOL 362 · 8 APRIL 1993



Cool Glow chemiluminescent system.

detecting products of dideoxy termination sequencing methods through chemiluminescence (*Reader Service No. 113*). The reagent is designed to replace radioisotopic labels with alkaline phosphatase activation of a chemiluminescent substrate. The Cool Glow detection system is compatible with current sequencing technologies. Instead of using radioactive tags, 5'-biotinylated primers are substituted in common, thermocycled, or multiplex sequencing protocols. After the reaction is complete, reaction products are simultaneously separated and transferred to a membrane with the Betagen AutoTrans direct transfer electrophoresis system or by manual blotting techniques. The membrane is then incubated at room temperature with Cool Glow reagents. In less than one hour, Betagen says that membranes are ready to expose to film; in less than 90 min, typical films are ready to read.

British Bio-technology announces the availability of new and improved versions of the *in situ* Instructor and the Digoxigenin Hybridization Detection Kits (*Reader Service No. 114*). The Instructor kit, which is designed to familiarize researchers with the *in situ* hybridization technique, now makes it possible to perform the technique in a day with no need for overnight hybridizations. The kit contains sufficient reagents for two complete runs of *in situ* hybridization, including the appropriate controls. Reagents have been combined so that the kit is more compact and easier to use. Colour-coded



In situ hybridization primer from BBt.

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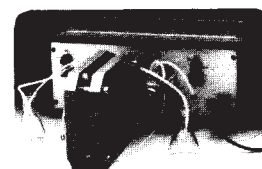
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dropper bottles have been included where possible to aid in the dispensing of solutions. The revealing reagents for the Digoxigenin Hybridization Detection Kit, previously in six separate bottles, have been reformulated and are now contained in a single bottle.

■ Reagents and consumables

The 1993–1994 Bios catalogue entitled *Mapping Strategies and Product Solutions* focuses primarily on the steps involved in positional mapping and functional mapping strategies and should be of interest to researchers mapping human genes or studying the relatedness of their nonhuman gene to the human genome (*Reader Service No. 115*). Chapter topics include chromosome localization, evolutionary relatedness, gene expression analysis (RNA products), DNA variation analysis by Southern blotting, DNA variation analysis by the polymerase chain reaction, subchromosome localization, polymorphic STS identification, chromosome analysis primers, microdissected chromosome DNA and molecular toolkits.

The new **DNA standards** from natural sources can be used as positive and negative controls in DNA amplification reactions. The preparations, which are available from Advanced Biotechnologies, are from purified DNA viruses, retroviral infected cells and uninfected cells and allow the researcher to amplify or test for any region of the genome that is present in natural infections (*Reader Service No. 116*). These preparations are not only virus-type specific but in many cases several strains of the same virus are available. The controls can also be used in nucleic acid hybridization reactions used in the detection of infectious agents, cloning viral sequences, establishing virus detection or quantitation assays for antiviral drug testing and quantitative polymerase chain reaction assays.

To meet all user needs, Synthecell/Vega will custom synthesize four different grades of **peptides** based on increasing levels of purity (*Reader Service No. 117*). The available grades include, unpurified, purified >90 per cent, >95 per cent and >98 per cent.

The company uses a variety of strategies in the production of peptides under good manufacturing practices, including solid- and liquid-phase synthesis utilizing either t-Boc or Fmoc chemistries. The quality-control documentation includes details of molecular weight, peptide content, amino acid analysis and HPLC purity level. Additional services include sequencing, conjugation, HF and TFA cleavage, HPLC purification, HPLC analysis, capillary zone electro-phoresis analysis, mass spectrum analysis and spectral modifications.

Sigma Electrophoresis is offering a complete line of **two-dimensional gel electrophoresis products**, including molecular weight markers, reagents and equipment (*Reader Service No. 118*). The



Sigma's 2-D electrophoresis line.

2-D marker mixture provides an easily observed diagonal line across the 2-D gel, whereas the carbamylated markers display approximately 20 spots on a line parallel to the isoelectric focusing (IEF) axis on IEF urea or 2-D gels.

Boehringer Mannheim has recently introduced an **RNase protection kit** that can be used to map transcription start and termination sites, determine the intron–exon structure of genes and to detect and quantify specific RNA sequences (*Reader Service No. 119*). The company says that the kit is

sensitive (0.1 pg RNA detection) and specific, and can be used with either radioactively-labelled or digoxigenin-labelled SP6 or T7 RNA transcripts as probes.

Ready-to-use Ultradig DNA *in situ* hybridization kits that are designed for the **detection and identification of micro-organisms** in paraffin sections, frozen sections and cytological specimens are now available from Kreatech Biotechnology (*Reader Service No. 120*). The DNA probes are labelled with digoxigenin and a one-step detection method is used. Taking only 40 min of hands-on time, Kreatech says that the kits can be used to visualize the DNA of papilloma viruses, polyoma viruses, herpes viruses, chlamydia, plasmodia and bacteria.

The new Triple Check 200-µl capacity **pipette tip** from BIO 101 is designed to allow visual confirmation of exact low-volume measurements of enzymes and other expensive or critical liquids (*Reader Service No. 121*). This specialty pipette tip, which fits most makes of pipettor, is marked with easy-to-see graduations at precise volumes or 0.5, 1, 2, 3, 4, 5, 10, 50, 100 and 150 µl. BIO 101 says that the pipette tip eliminates pipetting errors associated with differences in viscosity among various reagents. They are available in racks of 96 tips, with and without aerosol barriers, sterile and nonsterile. They are also available individually wrapped and sterile, as well as in bulk bags of 800.

BioDesign **dialysis tubing** is a thin, transparent tear-resistant cellulosic tubing that is designed to aid dialysis in biological research applications (*Reader Service No. 122*). The tubing has a nominal 8,000



BioDesign's dialysis tubing.

molecular weight cut-off and is available in 100-foot rolls or 12-inch cut strips. It is supplied in air-tight, moisture-proof plastic containers to maintain the flexibility of the tubing and ensure maximum shelf-life. Five diameters are available ranging from 6.4 mm to 49.5 mm. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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